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Review

Enzootic bovine leukosis and the risk to human health

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The bovine leukemia virus (BLV) was first reported as enzootic bovine lymphosarcoma in Eastern Europe in the late 19th century, highlighted by the presence of slightly yellow nodules in the enlarged spleen of cattle. It was believed to be an infectious disease because it spreaded through the herds. With changes observed in sensitivity of diagnostic techniques, the opinion that "BLV does not infect humans" starts to change after more than two decades. Several researches tried to link the BLV and human health in some studies in which BLV and human breast cancer have been shown. Studies about the possible routes of infection and to explain the genetic transformation processes in humans are raised. Multiple reports on this disease that link it to human health concluded on the need for a new mind set to understand relation between BLV and human health so as to improve the prevention, control and eradication of cattle herds.

Key words: Zoonotic disease, bovine leukemia virus, polymerase chain reaction (PCR), cancer.

INTRODUCTION

For years, the disease known as enzootic bovine leukemia (EBL) has been defined as a sickness limited to cattle, particularly dairy cattle. Researchers around the world have made significant efforts to demonstrate, without a doubt, that this disease is not related to any pathology in humans (Gilden et al., 1975; Donham et al., 1977; Burridge, 1981). With the emergence of new diagnostic technologies, the detection of a large diversity of pathogenic agents has improved, including the said disease. With the currently obtained results (Buehring et al., 2003; Buehring et al., 2007; Nikbakht et al., 2010; Mesa et al., 2013; Buehring et al., 2014; Buehring et al., 2015; Villalobos, 2016), doubts have once more emerged about whether this virus is capable of affecting human health. The objective of this review is to provide relevant information on this disease from its discovery in 1871 to 2016, including the history, classification and epidemiology, mechanisms of action of the virus to cause harm, cancer and its association with the virus, entry routes and mechanisms of cellular transformation, all of which cast new light on the implication of bovine leukemia virus (BLV) in human health. The definitive response on this subject still lacks a categorical response.

HISTORY

Bovine leukemia virus was first reported as enzootic bovine lymphosarcoma in Eastern Europe in the late 19th

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century, highlighted by the presence of slightly yellow modules in a splenomegaly observed in cattle; it was believed to be an infectious disease because it spread through herds (Leisering, 1871; Buehring et al., 2014). In 1917, its transmission through an infectious agent was demonstrated (Schwartz and Levy, 1994). Subsequently, Miller et al. (1969) used electron microscopy to demonstrate the presence of the viral particle within a lymphocyte in cows with lymphosarcoma; finally, using Koch's postulates, the causal association between the virus and enzootic bovine lymphosarcoma was successfully demonstrated (Olson et al., 1973; Olson, 1974). The first studies that showed evidence on the possible transmission of BLV in human cells and other species were conducted by Graves and Ferrer (1976), who obtained samples from cows infected with BLV in a state of persistent lymphocytosis that were cultivated in boundary cap (BC) cells; these samples proceeded to infect cell cultures of humans, simians (chimpanzees and rhesus), canines, sheep, goats and bats. In all of the cell cultures of the mentioned species, production of the complete virus was observed. The authors concluded that BLV can infect cells of the rhesus monkey, chimpanzees and humans, and it merits special attention in terms of its potential as a biohazard towards human beings.

Subsequently, Slavikova et al. (1987) isolated 60 clones of human myeloma B cells that were infected with BLV. Amongst the 60 clones evaluated, two harboured proviral sequences and the presence of virus proteins, confirming their expression ability in human cells *in vitro*. Altaner et al. (1989) obtained similar results using clones of foetal sheep cells (FLK) infected with virus to which human neuronal cells were exposed. The data showed that the neuronal cells could be infected by direct contact and the virus is capable of replicating itself; therefore, *in vitro*, these cells are susceptible and permissive to BLV. During the 1990s, Ursin et al. (1990) showed interest in the possibility that there is a risk to human health from BLV. These authors presented a prospective study in which a possible association between the consumption of cow's milk and cancer was investigated. The study followed 15,914 individuals for a period of eleven years, of whom 1,422 were diagnosed with cancer. The types of cancer evaluated were cancer of the lip, mouth, throat, oesophagus, stomach, colon, rectum, pancreas, larynx, respiratory tract, breast, cervical-uterine, ovarian, prostate, kidney, bladder, melanoma, skin cancer, thyroid, lymph organs, multiple myeloma and leukaemia. Risk factors considered were active tobacco consumption and ex-smokers and alcohol, meat, egg and coffee consumption. Although the investigation did not identify an association between milk consumption and total incidence of cancer, there was a strong association between milk consumption (two cups daily) and cancer in lymph organs, particularly lymphosarcoma, the same condition that is present in cattle. The study also found a slight positive association with kidney cancer and female reproductive organs, except for cervical-uterine cancer. In 1990, an increase in cancer was observed by Davis et al. (1990) in the agricultural population of several countries (Germany, Italy, United States, Japan, England and Wales), particularly in the lymphatic and haematopoietic system compared to the general population; thus, special attention was demanded on this topic (Blair et al., 1992). However, in other studies, such as those by Fritschi et al. (2003) in meat workers or reports by Sellers et al. (2008) on the risk of consuming unpasteurized milk, there have not been consistent results on the risks of contracting any type of cancer in particular.

CLASSIFICATION, HOSTS AND REPLICATION STRATEGY OF THE VIRUS

BLV (Retroviridae family; Orthoretrovirinae subfamily, *Deltaretrovirus* genus) is an exogenous retrovirus, responsible for EBL, which is the most common neoplastic disease in cattle worldwide (Schwartz and Levy, 1994; Dequiedt et al., 1999; Beyer et al., 2002; Moratorio et al., 2013). BLV is related to *human T-lymphotropic virus* types 1, 2 and 3 (HTLV 1, 2 and 3) and *primate T-lymphotropic virus* types PTLV 1, 2 and 3 (Gelmann et al., 1983; Tanaka et al., 1990; Heenemann et al., 2012). Infection is transmitted horizontally, through the transfer of infected cells by direct contact, ingestion of colostrum, milk and possibly through bloodsucking insects (Ferrer et al., 1978; Gillet et al., 2007). Vertical transmission (cow to calf) has also been demonstrated transplacentally (Ferrer, 1979; Van der Maaten et al., 1981; Romero et al., 1983; Lassauzet et al., 1991; Hübner et al., 1997). Although the virus has been demonstrated to have cattle as its main host, infection also occurs in buffalo and capybaras (Schwartz and Levy, 1994). Sheep and goats have been experimentally infected and have been routinely used in the investigation of BLV, with the particularity that goats develop cancer faster than cattle (Schwartz and Levy, 1994; Gillet et al., 2007; Merimi et al., 2007); therefore, it is proposed that the mechanisms of leukemogenesis in cattle and goats are likely different (Graves and Ferrer, 1976; Djilali and Parodi, 1989).

The propagation of BLV in the host occurs through two distinct processes. In the first process, the infectious cycle results from the virion coupling with the target lymphocyte, entry of the single-stranded viral RNA, reverse transcription and integration as a provirus into the host genome (also known as the infectious cycle). The second replication strategy depends on the management of cellular proliferation using viral regulatory proteins such as *Tax*. The two routes of viral replication produce a group of infected cell populations composed of distinct clones (Gillet et al., 2013). In the same study, Gillet et al. (2013) also demonstrated that BLV is initially directed to

transcribed regions of the genome for its integration; afterwards, a massive selection of clones is produced during primary infection, disfavouring proviruses located near genes; however, the abundance of long-term clones benefits transcriptional activity of the genomic region surrounding the provirus.

EPIDEMIOLOGY AND DIAGNOSIS OF THE EBL

The Guaymí and Guabalá are breeds reported in 2010 (Villalobos et al., 2010) and have been the subject of various studies in order to preserve them and use them for the production of milk and meat (Delgado et al., 2012; Martinez et al., 2012). With the emergence of an outbreak of EBL in native cattle in Panama in 2011, new diagnostic protocols were developed in order to replace the agar gel immunodiffusion (AGID) by better methods such as blocking enzyme-linked immunosorbent assay (ELISA) and nested polymerase chain reaction (PCR), and the development of genetic studies of disease resistance (Villalobos and Gonzalez, 2015). With the recent reports of the virus in human beings, a new line of research was created by the use of gene markers env, gag and *Tax* in human lymphocytes (Villalobos, 2016).

THE ZOONOTIC POTENTIAL OF BLV

In a study conducted by Buehring et al. (2001) under the premise that BLV is present in many of the meat products and milk on the market and because the incidence of breast cancer is higher in countries with a high consumption of food products from bovine species, the authors found that many humans possess antibodies against BLV, particularly the envelope glycoprotein (gp51) and capsule protein (p24), suggesting the possibility of infection with the virus. These same authors, using immunohistochemistry and PCR *in situ* in patients diagnosed with cancer and subject to surgical excision, showed that most mammary tissues studied presented evidence of a proviral genome of BLV, and four of the 27 samples were positive for the virus capsule protein.

With changes observed in low sensitivity techniques, such as complement fixation or agar gel immunodiffusion (AGID) in the 1970s and 1980s (Gilden et al., 1975; Donham et al., 1977; Burridge, 1981), compared to more modern techniques such as the enzyme linked immunosorbent assay (ELISA) and immunoblotting, the opinion that had prevailed for more than two decades that “BLV is not transmissible to humans and no disease in humans has been attributed to BLV” could be changing. In a study performed at the University of Berkeley, California, by Buehring et al. (2003) based on serum samples of 257 people, four isotypes of antibodies were used (IgG_1, IgM, IgA and IgG_4) to detect the capsule (p24) antigen of BLV. At least one reactive isotype to the protein was detected in 74% of the evaluated population. Although the investigation did not conclude there was infection to humans, it again opens the debate on the possibilities of additional studies with techniques such as real time PCR because of the possibility that the reaction was in response to denatured antigens from heat action in ingested foods. Conversely, Lee et al. (2005) investigated the possible relationship between leukaemia, lung cancer and meat consumption in Korea, using new primer sets of the envelope gene; the results were negative, indicating that virus infection did not occur in any of the cases.

To provide evidence of possible zoonotic behaviour of BLV, Ochoa-Cruz et al. (2006) selected 56 cases diagnosed with ductal carcinoma, of which the immunoperoxidase assay was applied for the purpose of detecting glycoprotein gp51 of BLV in the cytoplasm of tumour cells. The technique showed that 7% of samples were positive for gp51, demonstrating the presence of BLV in humans with the ability to produce viral protein; the appearance of this molecule implies that the active provirus inserted in the genome is capable of producing structural viral proteins to assemble progeny. Subsequently, Buehring et al. (2007) used *in situ* PCR with primers of the *Tax* region of BLV to evaluate 213 samples of mammary gland tissue sections fixed with formalin distributed in 110 women with breast cancer and 103 controls (women without a history of breast cancer). The investigation showed positive reactions to BLV in 59% of the cases of women with breast cancer and in 29% of the control cases. Amongst the samples from women with breast cancer, 69% showed proviral BLV DNA in the accompanying non-malignant mammary epithelium, suggesting that the development of cancer may be an exceptional case, delayed within a population of cells infected with BLV in mammary tissue (field effect). These data provide a first promising step in the establishment of a causal role of BLV in human breast cancer.

Another investigation in which serological (ELISA) and genomic (PCR) techniques were used was conducted by Nikbakht et al. (2010) in the School of Veterinary Medicine of the University of Tehran; this study analysed 454 samples of human patients without any clinical symptoms in particular. Based on the serological test, 57 patients (12.5%) were diagnosed as positive. For the PCR test, 77 patients were evaluated (57 positive and 20 negative to the ELISA test). It was possible to isolate provirus in 12.3% of the 57 positive ELISA samples. Although the examination revealed the presence of provirus, the authors remained cautious in the conclusions because the provirus may not necessarily be integrated into the genome but rather be a non-integrated element (episome); in the latter case, people may not be actively infected. A study similar to that conducted by Buehring et al. (2007) was developed by Mesa et al. (2013) in Colombia with 106 mammary tissue samples from female patients (53 patients were positive for breast

cancer and 53 were negative). A PCR analysis was performed on these patients to detect the gag segment of BLV. Of the analysed samples, 35% of the patients positive for breast cancer were positive for gag amplification and 45% of the patients were negative. Given the discovery of antibodies against BLV in humans, Buerhing et al. (2014) used human mammary tissue for BLV infection tests using liquid phase PCR (L-PCR), sequencing, in situ PCR and immunohistochemistry (IHC). The studies focused on mammary tissue because in the original host, bovine cattle, BLV DNA and p24 protein are found in greater abundance than in lymphocytes. The findings of this investigation conclude that there is evidence showing that the BLV DNA and protein found present a high likelihood of constituting the *in vivo* presence of BLV in humans. Subsequently, Buehring et al. (2015) conducted a study on the cause-effect relationship between BLV and breast cancer in 239 patients with positive and negative records, using anatomopathological studies of mammary tissue. The presence of the virus was observed in 59% of malignant tissues, 38% of tissues with premalignant changes and 29% of tissues from normal controls. The study concluded that there is a highly significant cause-effect relationship. However, the authors also noted that one control study is not conclusive on its own, and validation from other investigators is required. In a randomized study conducted in a region in Panama by Villalobos (2016), 20 patients were sampled anonymously in order to detect the gag gene of enzootic bovine leukosis virus and 75% of them were positive. A larger project is currently being conducted with the aim of increasing the number of human patients and the virus markers like *Tax*, env and pol. Furthermore, a prospective study is required that demonstrates that viral infection precedes the development of cancer to support the idea of causality of the virus towards breast cancer. In light of the possible public health consequences of BLV in humans, future research should address how humans are infected by BLV, the frequency with which BLV infection is produced in different populations and whether the virus is associated with disease in humans.

ROUTES OF BLV ENTRY INTO THE HUMANS

As the presence of the virus within humans has been successfully demonstrated, whether by indirect methods such as ELISA and immunoblotting or direct methods such as PCR, several entry routes have now been proposed, such as direct contact with animals or animal products. The consumption of unpasteurized milk, artisanal cheeses and improperly cooked meat could be entry vehicles of the virus in populations likely to be exposed in rural areas such as Panama, Peru, Mexico and the United States, where a very common practice is ingesting milk from cows. For example, a study conducted in the United States by Oliver et al. (2009) shows that between 35 and 60% of families and employees at farms ingest unpasteurized milk, and cattle herds infected with BLV are found throughout the world. In the United States, close to 38% of cattle herds, 84% of dairy herds and 100% of herds of large-scale dairy operations are infected (USDA, 1999, 2008). The detection of antibodies due to the consumption of foods derived from the bovine species had previously been reported by Barnes et al. (1988), particularly the reactivity from isotypes IgG2 and IgG4 towards antigens of bovine milk. Buehring et al. (2003) reported using the isotype IgG4 in the reactivity to BLV. Notably, viral particles from BLV denatured by pasteurization or heat can cause reactivity from the human immune system; however, it is not possible to differentiate them from un-denatured viral particles (Buehring et al., 2003). Another possible entry route could be injection of biological products contaminated by BLV, for example, with the development of anti-hemoparasite vaccines (Callow et al., 1997) and the incidence of many vaccines contaminated with BLV, as reported by Rogers et al. (1988), which forced the implementation of more rigorous diagnostic techniques against BLV in Australia. However, there are no reports of BLV contamination or production of the virus in *in vitro* cell lines for vaccines (Buehring et al., 2003). Furthermore, epithelial cells are identified as the entry route of the virus, through a genome integration process and the recognition of similar receptors to those of cattle (BVLRcp1) or through the interference of other unidentified molecules such as IgM, CD5+ and CD11b integrins, similar to experimental infection in studies on goats (Mesa et al., 2013). Importantly, once a zoonotic virus enters the human population, the majority are capable of dispersing amongst the population, a process that constitutes the most severe threat for human health (Christou et al., 2011). On this line of thought, BLV is known for crossing towards other species easily; the virus naturally infects capybara, Zebu cattle water buffalo, and it has experimentally infected sheep, goats, pigs, rabbits, rats and chickens (Schwartz and Levy, 1994). Moreover, human cells (fibroblasts) are susceptible to infection with BLV *in vitro* (Diglio and Ferrer, 1976).

MECHANISM OF LEUKEMOGENESIS BY BLV

Leukemogenesis mechanisms (induction of leukemia) through animal retroviruses that belong to the *Alpharetrovirus* and *Gammaretrovirus* genera induce tumour production by two mechanisms: activation of a viral oncogene or insertion of a gene from the cell, such as a proto-oncogene (Weiss et al., 1985). However, deltaretroviruses such as BLV lack a known oncogene (Sagata et al., 1984). Most of the studies on leukemogenesis induced by BLV have focused on the Tax protein because it is considered a potent

transcriptional activator of viral gene expression. In addition to its function as a transcriptional activator, the Tax protein induces the immortalization of fibroblasts of the rat embryo (Willems et al., 1990, 1998). This ability to induce immortalization may be the first step in the transformation process mediated by BLV. However, once the cattle are infected and during the latent period, the expression of BLV is blocked at the transcriptional level (Kettmann et al., 1982; Lagarias and Radke, 1989). Such repression appears to be very important for the escape of BLV from the immune surveillance system of the host, and subsequently only a small proportion of infected animals would rapidly develop the terminal stage of the disease (Gillet et al., 2007). In fact, transcription of the BLV genome in fresh tumour cells or in peripheral mononuclear cells (PBMCs) in fresh blood of infected individuals is almost undetectable by conventional techniques (Kettmann et al., 1982; Tajima et al., 2003; Tajima and Aida, 2005).

LEUKEMOGENESIS AND *PX* REGION

All retroviruses possess the genes gag, pro, pol and env, which encode the internal structural proteins, viral protease, reverse transcriptase and envelope glycoproteins of the virion, respectively, and are essential for the production of infectious viral particles. The genes are flanked by two identical long terminal repeats, LTRs (Alfaro et al., 2012). Although the genome sequences of BLV and HTLV-1 differ, they have a sequence in common called pX that is located between the env gene and the 3'LTR region that encodes a regulatory gene. In both viruses, the regulatory proteins Tax and Rex are encoded in the pX region. The R3 and G4 proteins are encoded in the pX region of BLV, whereas $p12^{I}$, $p13^{II}$ and $p30^{II}$ are encoded in the pX region of HTLV-1 (Sagata et al., 1984; Franchini et al., 2003). The pX sequences do not originate from the host cells and thus it is not an oncogene. In both BLV and HTLV-1, the Tax protein acts as an activator of transcription with oncogenic potential, and Rex interferes with the exportation of messenger RNA of both viruses from the nucleus (Derse, 1987; Willems et al., 1987; Felber et al., 1989; Katoh et al., 1989; Willems et al., 1990; Kashanchi and Brady, 2005; Matsuoka and Jeang, 2011). In cattle, the R3 and R4 proteins contribute to the maintenance of a high viral load (Willems et al., 1994; Florines et al., 2007). Furthermore, $p12^{I}$ and $p13^{II}$ proteins from HTLV-1 are similar in some functions to R3 and G4, respectively. $p12^{I}$ resembles R3 in that both are maintained in the nucleus and contribute to infectivity of the virus (Collins et al., 1998; Gillet et al., 2007); the $p13^{II}$ protein resembles the G4 protein because both bind to the farnesyl pyrophosphate synthetase, which farnesylates ras (Lefebvre et al., 2002) in addition to promoting ras dependent apoptosis (Hiraragi et al., 2005). Similarly, suppressions have been observed in the sequences of gag, pol and env in all of the BLV-positive panel samples, and the presence of LTR sequences and Tax in these samples is consistent with the results reported for HTLV-1, which are associated with escape from immune surveillance (Kamihira et al., 2005; Buehring et al., 2014).

MECHANISMS OF CELLULAR TRANSFORMATION

Given the reports of the presence of BLV found integrated to the genome or as an episome in humans in the United States by Buehring et al. (2007), in Iran by Nikbakht et al. (2010) and in Colombia by Ochoa-Cruz et al. (2006), Mesa et al. (2013) and Buehring et al. (2014), possible mechanisms of transformation in mammary tissue must be proposed. These include involvement of the *Tax* gene in oncogenic processes *in vivo* and *in vitro*, such as viral transcription and increased expression in the proportion of the Bcl-2 protein (a proto-oncogene) over its protein homologue, Bax, which are related to resistance to apoptosis and the production of leukaemia in infected cows (Takahashi et al., 2005). A similar mechanism of resistance to apoptosis and chronic lymphocytic leukaemia occurs in humans (Pepper et al., 1997). Some authors mention that in the BLV genome, there is no preference for a particular site in the host genome (Murakami et al., 2011), and BLV could integrate itself in active sites associated with the control of cell division (Fulton et al., 2006; Klener et al., 2006). However, Gillet et al. (2013) demonstrated that BLV and HTLV-1 have surprisingly similar genomic regions where it is expected that the provirus of both viruses is inserted with a greater likelihood in their respective hosts. These insertion sites are the regions transcribing Pol II (polymerase) and Pol III, the regions close to the CpG islands, tRNA genes (transfer RNA) and tRNA pseudogenes. Similarly, in a study conducted by Elemans et al. (2014) on the mortality rate of cytotoxic T lymphocytes CD8+ (CTL) against infection of BLV and HTLV-1, both viruses are in the lowest range observed in the literature. This similarity could lead to finding similar mechanisms of action of BLV in humans.

Actions of the virus on the block of tumour suppressor and apoptosis genes could be a possible cause, as Melana et al. (2002) reports in breast cancer in the case of mouse mammary tumour virus (MMTV). Mutations of the p53 gene have been reported in 20% of women with breast cancer, and 30% of malignant processes in humans have been attributed to mutations of the oncogene ras (Javier and Butel, 2008).

Given the possibilities of action of the virus in humans, cases of benign, pre-malignant and malignant cell transformations and cases of latent virus presenting in apparently healthy patients could possibly be found without presenting changes in tissue, as shown in several previously mentioned reports and in the bovine species

or other retroviruses such as HIV and HTLV (Mesa et al., 2013). An additional finding has been made in recent years by Kinkaid et al. (2012), who demonstrated the presence of micro RNA (miRNA) in BLV. These miRNAs are small regulatory sequences encoded by most eukaryotic cells and some viruses that collectively have DNA type genomes. The miRNA type BLV-miR-B4 has been identified in BLV, which is transcribed by RNA polymerase III (pol III). The BLV-MiR-B4 shares partial identity of its sequences. It also shares target sequences with the miRNA of the bovine host (miR-29), and because the overexpression of miR-29 is associated with neoplasias of B lymphocytes that resemble tumours associated with BLV, a possible mechanism is suggested that contributes to the tumour genesis of BLV, similar to the participation of cell transformation in humans. Currently, six viruses are causally associated with cancer in human patients: The Epstein-Barr virus (EBV), human T-lymphotropic virus type 1 (HTLV 1), hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV) and human herpes virus 8 (HHV-8). However, given the growing research, using more advanced techniques such as ELISA, immunoblotting, nested PCR and RT-PCR, BLV is a new candidate to join the list of potentially hazardous viruses to human health and particularly cancer of the mammary gland (Rees, 2012). Other viruses together with BLV have also been proposed as potentially hazardous to human health, such as MMTV and cytomegalovirus (Lawson, 2006; Mason et al., 2011); thus, a change of thinking on the relationship between cancer and viruses is necessary. There is research in progress that could resolve various hypotheses that remain unsupported; however, as long as new lines are opened with methodologies that allow a better understanding of the action of the virus, particularly BLV and cancer in humans, new and better diagnostic, prevention and control methodologies of these diseases will continue to develop. Breast cancer occupies a significant position worldwide in terms of morbidity and mortality. Between 5 and 10% of all breast cancer cases are associated with hereditary factors. The rest are associated with other factors such as infections, of which 8% of the malignancy is reported in developed countries and an impressive 23% in developing countries.

CONCLUSION

Considering the scientific facts on the high prevalence of the EBL virus in countries such as the United States, the consumption of milk, meat and animal by-products positive for the disease, the constant exposure to the virus, the immune response against it and, ultimately, the demonstrated presence of the virus in the human genome, it is clear that there is a real potential risk. It is necessary to direct greater investment in research; rethink a new vision on the risks to human health; and develop programmes of diagnosis, prevention, control and eradication of the virus, particularly in countries with high prevalence. These steps may result in less exposure to the virus and a consequent reduction of the risk that it becomes, from the effects of evolution, a true zoonosis.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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