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Parasite Strain Specificity of Precursor Cytotoxic T Cells in Individual Animals Correlates with Cross-Protection in Cattle Challenged with *Theileria parva*

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Class I major histocompatibility complex-restricted parasite-specific cytotoxic T lymphocytes (CTL) are known to be a major component of the bovine immune response to the protozoan parasite *Theileria parva*, but formal proof for their role in protection of cattle against infection with *T. parva* has been lacking. Animals immunized with one stock of *T. parva* show variations in the degree of protection against heterologous challenge and also in the parasite strain specificity of their CTL responses. The present study investigated the relationship of strain specificity of CTL responses and cross-protection in an effort to verify the role of CTL in protection. The parasite strain specificity of the CTL responses generated in 23 cattle immunized with either of two immunologically distinct parasite populations was examined, and the susceptibility of individual cattle to challenge with the heterologous parasite population was determined. The frequency of stock-specific or cross-reactive CTL precursor cells (CTLp) in individual animals was measured by a limiting-dilution microassay. A proportion of animals immunized with either parasite exhibited cross-reactive CTLp, whereas CTLp detected in the remaining animals were specific for the homologous parasite. On challenge with the heterologous stock, those animals with cross-reactive CTLp were solidly protected while those with strain-specific CTLp showed moderate to severe reactions, although many of them recovered. The finding of a close association between strain specificity of the CTL response and protection against challenge provides strong evidence that CTL are important in mediating immunity.

Class I major histocompatibility complex (MHC)-restricted cytotoxic T-lymphocyte (CTL) responses have been demonstrated to occur in a number of intracellular protozoan parasitic infections of humans and animals. These responses have been most extensively studied in human and rodent infections with *Plasmodium* species (12, 13) and in cattle infected with *Theileria parva* (18). Although there is evidence that CTL responses can confer protection in murine models of malaria, the precise role of these responses in immunity to the natural human and bovine diseases has yet to be determined. *T. parva* causes an acute and usually fatal disease of cattle that occurs throughout a large part of eastern, central, and southern Africa (3). The parasite at the sporozoite stage infects lymphocytes after inoculation by the tick vector and induces them to proliferate; simultaneous division of the schizont stage parasite and the infected cell results in a clonal expansion of parasitized cells (10), which become disseminated throughout the lymphoid system and certain nonlymphoid organs such as the lungs. The capacity to establish continuously growing cell lines by infecting lymphocytes with *T. parva* in vitro has greatly facilitated studies of parasite-specific T-cell responses. The parasite, therefore, offers a well-defined model system to study the induction of CTL responses to protozoan infections and to examine their role in immunity.

Cattle can be immunized against *T. parva* by infection with sporozoite stabilates of the parasite and treatment with oxytetracyclines. A shortcoming of this method is that immunity induced by one stock of the parasite does not usually extend to all other stocks (20). The basis of this stock heterogeneity is complex, since failure to protect is often not reciprocal between stocks and usually involves only a proportion of animals. Hence, studies with the Muguga and Marikebuni stocks of the parasite have shown that animals immunized with the Marikebuni stock are protected against challenge with the Muguga stock whereas a proportion of animals immunized with the Muguga stock remain susceptible to challenge with the Marikebuni stock (11, 17).

There is good evidence that the immunity induced by infection and treatment is directed against schizont-infected lymphoblasts and is mediated by cellular rather than humoral immune responses (14). Remission of infection following immunization or challenge is associated with the detection of potent CTL responses specific for parasitized lymphoblasts (4, 18), and these cells have been shown to be class I MHC restricted, $CD8^+$, and, in some instances, parasite strain specific (7, 18). The results of recent adoptive transfer studies utilizing monozygotic twins have demonstrated that $CD8^+$ CTLs derived from donor efferent lymph draining at the site of challenge with *T. parva* protected the recipient twin against parasite infection, documenting a direct role for CTLs in protective immunity (15). Despite the temporal relationship of the CTL responses with clearance of infection and their observed parasite strain specificity, a correlation between the parasite strain specificity of CTL and cross-protection has not been established. This has been due partly to the lack of a sensitive assay for measuring CTL responses in immune cattle prior to challenge and partly to parasite strain heterogeneity within some stocks of *T. parva*. In the study reported herein, we have used

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a limiting-dilution (LD) analysis assay (23) to measure the strain specificity of CTL responses in cattle immunized with the Muguga stock or a clone derived from the Marikebuni stock of *T. parva* and examined the susceptibility of these animals to challenge with the heterologous parasite. These experiments have demonstrated a close correlation between the strain specificity of CTL responses and the capacity to resist heterologous challenge.

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MATERIALS AND METHODS

Animals. Twenty-three Boran (*Bos indicus*) steers or female animals, aged between 8 and 42 months, were used. All cattle were reared indoors under tick-free conditions and at the outset of the experiments were clinically normal and negative for antibodies to *T. parva*.

Parasites. The ILRAD 3087 sporozoite stabilate of *T. parva* (Muguga) and stabilate 3219, a cloned population of the Marikebuni stock, were used for animal immunizations and establishment of cell lines. The latter stabilate was derived by tick pickup from two animals infected with a twice-cloned (by LD) parasitized cell line obtained by in vitro infection of lymphocytes with *T. parva* (Marikebuni) sporozoite stabilate 3014. Previous studies involving immunofluorescent staining (16) and Western blotting (immunoblotting) (25) with schizont-specific monoclonal antibodies (MAb) (19) and DNA restriction fragment length analysis with *T. parva*-specific DNA probes (2) distinguished between the Muguga and Marikebuni stocks and also detected heterogeneity within the Marikebuni stock (25). Parasite-specific MAb 2 and 3 identify Muguga, and MAb 15 and 16 identify Marikebuni (19). MAb BT5/2.2.5 identifies a schizont surface antigen (19, 21) that differs in molecular weight (M_r) between stocks of *T. parva* and between subpopulations of the Marikebuni stock. For establishment of cell lines, sporozoites were obtained from salivary glands of ticks fed on cattle infected with either stabilate and used to infect peripheral blood mononuclear cells in vitro (6).

Immunization and challenge of cattle. Cattle were immunized with the appropriate population of parasites by simultaneous inoculation of a lethal dose of cryopreserved sporozoite stabilate and 20 mg of long-acting tetracycline (Embacycline LA; May and Baker, Ltd., Dagenham, England) per kg of body weight, as described previously (20). The animals were challenged 5 to 8 months after immunization with a previously determined 100% lethal dose of sporozoite stabilate. All inoculations were subcutaneous on the right side of the neck. Cattle were monitored for parasitosis (percent schizont-infected lymphocytes per total number of lymphocytes in the field) by microscopic examination of smears of aspirates of local and contralateral lymph nodes stained with fluorescein isothiocynate-conjugated antiserum to *T. parva* schizonts. Examination for parasitosis and measurement of rectal temperature were performed daily starting from 5 days after inoculation until recovery or treatment of the animal. Parasitosis was graded as mild (<1%), moderate (1 to 10%), or severe (>10%). A rectal temperature of 39.5°C or higher, associated with schizont parasitosis, was considered a pyrexic response. Recovery of animals was defined as disappearance of schizonts from lymph nodes, a rectal temperature below 39.5°C, and a clinically healthy appearance.

LD culture for assay of CTLp. An LD culture system employing parasitized cell lines as stimulator and target cells for generation and assay of cytotoxic cells, as described previously (23), was used to measure CTL precursors (CTLp) in peripheral blood mononuclear cells derived from immunized cattle. All cultures were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) of a selected batch of heat-inactivated fetal calf serum (Gibco Ltd., Paisley, Scotland), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 50 μg of gentamicin per ml. Medium for culture of parasitized cell lines also contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer at pH 7.2.

Cytotoxicity assays. Cytotoxicity generated in the LD microcultures after 7 days in culture was measured by an ^{111}In release assay performed in V-bottom 96-well assay plates (Greiner, Nuertingen, Germany) as described previously (22). Contents of individual wells were split into two aliquots and tested with autologous target cells infected with the Muguga stock or clone 3219 of the Marikebuni stock. Wells were scored as positive if they had isotope release values three times the standard deviation above the mean of the background release values. The natural logarithm of the fraction of negative wells was plotted against the number of responder cells per well, and the best linear fit to the data was obtained by computerized maximum-likelihood analysis (5). The frequency of CTLp was then calculated directly from the gradient of the plot. Statistical evaluation of the cytotoxicity data by this method in previous studies (23) had shown that data conformed to the Poisson model of single-hit kinetics (24).

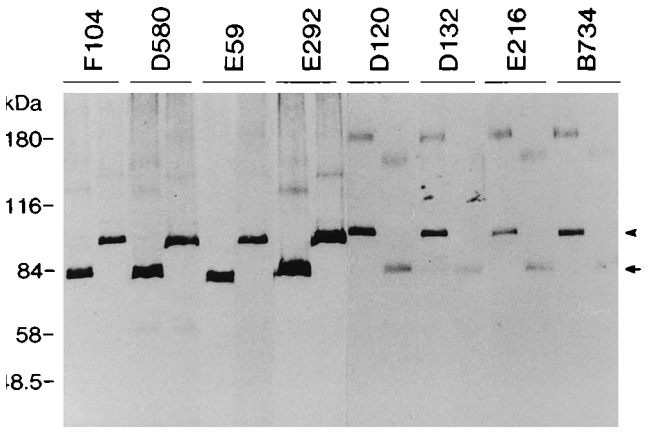

FIG. 1. Western blot analysis with MAb BT5/2.2.5 of lysates of 16 of 46 cell lines infected with the Muguga stock (➤) or clone 3219 of the Marikebuni stock (→) of *T. parva* is shown. The methodology followed in the analysis of these cell lines was essentially as reported before (8). Designations above lanes refer to some of the cattle used in the study.

RESULTS AND DISCUSSION

The interpretation of results of previous studies examining the relationship of strain specificity of CTL responses and cross-protection following *T. parva* infection has been problematical because of the heterogeneity of the parasite stocks used for challenge and the fact that parasitized cell lines used to assay CTL activity often represent a selected component of the parasite stock. Thus, on the basis of analyses with MAb and DNA probes, at least five distinct parasite populations are identifiable within the Marikebuni stock (2, 25). Moreover, some of these populations are recognized as different by parasite-specific CTL clones (8). In established cell lines infected with the Marikebuni stock, one component of the stock usually predominates, presumably as a result of selection, and it is not uncommon for cell lines established on the same day, with the same batch of sporozoites, from lymphocytes of different cattle, to contain different parasites (16a). Consequently, assays of CTL responses to the Marikebuni stock using such cell lines may detect only a component of the in vivo response and the results obtained with different animals are not necessarily comparable. Moreover, when the Marikebuni stock is used for challenge in cross-protection experiments, different components may be responsible for breakthrough infections in different individual animals. Hence, a cloned population of *T. parva* (Marikebuni 3219) parasites was used in the present study. To establish that this population of parasites was indeed homogeneous and had not changed during tick passage, and to compare it with the Muguga stock, parasites within the cell lines established from the animals used in the study were analyzed with parasite-specific MAb. MAb 2 and 3 reacted at titers of 1:3,200 or greater with all of the Muguga-infected cell lines but gave no specific staining (titer < 1:200) with any of the lines infected with Marikebuni 3219. Conversely, all of the cell lines infected with Marikebuni 3219 but none of the lines infected with Muguga reacted with MAb 15 and 16. MAb BT5/2.2.5 was used to analyze Western blots of cell lysates from all of the cell lines. Representative results are shown in Fig. 1. In all of the Marikebuni 3219 lines the antigen had an M_r of 85,000, whereas in the Muguga lines it had an M_r of 92,000. The reactivity of the MAb with the Marikebuni 3219 lines and the M_r of the antigen detected in these lines by MAb BT5/2.2.5 are identical to those observed with the original

TABLE 1. Pyrexic and parasitosis reactions in 17 *T. parva* (Muguga)-immune and 6 susceptible cattle following challenge with *T. parva* (Marikebuni 3219)[a]

Animal	Pyrexia		Parasitosis				Outcome[b]
	Day of onset	Duration (days)	Day of onset	Duration (days)	Severity in:		
					Local LN	Contralateral LN	
Susceptible							
E44	8	11	8	11	3+	3+	Treated
E48	10	12	10	11	3+	3+	Treated
E50	9	14	9	13	3+	3+	Treated
F153	8	13	10	12	3+	3+	Treated
F154	8	13	9	12	3+	3+	Treated
F186	6	11	7	10	3+	3+	Treated
Immunized							
D120	12	1	12	1	1+	NPS	Recovered
D580	12	3	12	1	1+	NPS	Recovered
D761	—	—	10	2	1+	NPS	Recovered
E49	—	—	11	1	1+	NPS	Recovered
E98	—	—	11	2	1+	NPS	Recovered
E292	8	4	10	3	1+	NPS	Recovered
D132	10	6	12	9	2+	2+	Recovered
D717	7	10	9	17	2+	2+	Recovered
E45	11	13	11	10	2+	2+	Recovered
E81	7	11	8	22	2+	2+	Recovered
E118	12	14	12	21	2+	2+	Recovered
E216	8	19	8	12	3+	2+	Recovered
E249	7	12	8	9	2+	2+	Recovered
E260	7	9	8	12	2+	2+	Recovered
F25	8	11	9	9	3+	2+	Recovered
F304	7	11	8	12	3+	3+	Treated
F339	7	11	8	9	3+	3+	Treated

[a] Abbreviations and symbols: LN, lymph node; NPS, no parasites seen; —, no pyrexia detected. 1+, <1% parasitosis (mild); 2+, 1 to 10% parasitosis (moderate); 3+, >10% parasitosis (severe).
[b] Severely reacting cattle were treated on day 16 of parasitosis.

cloned cell line prior to tick passage (data not shown). These results confirm that the Marikebuni 3219 parasite is antigenically distinct from the Muguga stock and, as far as can be determined, is a homogeneous population.

To determine the cross-immunizing potentials of the Muguga stock and clone Marikebuni 3219, two groups of cattle were immunized and subsequently challenged with the heterologous parasite. Seventeen cattle immunized against *T. parva* (Muguga) and six susceptible controls were challenged with Marikebuni 3219. Similarly, five of six animals immunized against Marikebuni 3219, along with two susceptible controls, were challenged with *T. parva* (Muguga). As shown in Tables 1 and 2, all of the challenge controls had severe reactions that required treatment, whereas the severity of the reactions in the immunized animals varied markedly. Six of the 17 Muguga-immunized animals had mild or inapparent clinical reactions with transient parasitosis that was confined to the local lymph node and did not exceed 1% (Table 1). The remaining 11

TABLE 2. Pyrexia and parasitosis in five *T. parva* (Marikebuni 3219)-immune and two susceptible cattle following challenge with *T. parva* (Muguga)[a]

Animal	Pyrexia		Parasitosis				Outcome[b]
	Day of onset	Duration (days)	Day of Onset	Duration (days)	Severity in:		
					Local LN	Contralateral LN	
Susceptible							
G337	9	13	6	14	3+	3+	Treated
G338	8	8	6	11	3+	3+	Treated
Immunized							
D540	—	NA	10	1	1+	ND	Recovered
E59	8	10	10	9	3+	ND	Recovered
E82	8	12	7	13	3+	ND	Recovered
E212	9	2	8	1	1+	ND	Recovered
E259	7	1	NA	NA	NPS	ND	Recovered

[a] Abbreviations and symbols: NPS, no parasites seen; LN, lymph node; —, no pyrexia detected; NA, not applicable; ND, not done. 1+, <1% parasitosis (mild); 2+, 1 to 10% parasitosis (moderate); 3+, >10% parasitosis (severe).
[b] Severely reacting cattle were treated on day 16 of parasitosis.

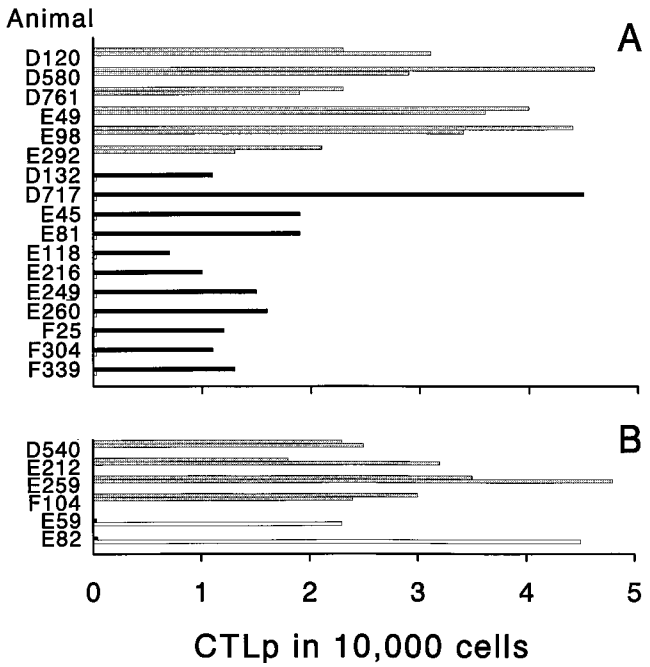

FIG. 2. Strain specificity of CTLp generated in individual cattle immunized against *T. parva* (Muguga) (A) or *T. parva* (Marikebuni 3219) (B) is shown. With the use of the LD microassay described in Materials and Methods, the frequencies of CTLp recognizing autologous lymphoblasts infected with the Muguga stock (■), clone 3219 of the Marikebuni stock (□), or either parasite stock (▨) were obtained. In all of the experiments reported herein, the correlation coefficient (r^2) values of CTLp frequencies were always greater than 0.85 (23). Values of frequencies less than 1:20,000 were considered undetectable, since the responder cell input of 2×10^4 per well is the upper detection limit of the assay.

animals exhibited prolonged reactions with fever lasting 6 to 19 days and parasitosis detectable for 7 to 22 days. Parasitosis was detected in both local and contralateral lymph nodes in all of the animals and attained maximal levels of 5 to 44%. Two of these animals required treatment, while the others recovered naturally. Of the five Marikebuni 3219-immunized animals that were challenged, three had mild reactions while the remaining two cattle suffered severe reactions but eventually recovered (Table 2). On the basis of these findings, it was concluded that the 6 Muguga-immunized animals that had mild reactions following challenge with Marikebuni 3219 were protected while the remaining 11 animals were partially or fully susceptible. Similarly, three of the animals immunized with Marikebuni 3219 were considered to be protected while two were susceptible to the Muguga challenge.

To evaluate the relationship between the specificity of CTLs and protection against heterologous challenge, the strain specificity of CTLp in peripheral blood mononuclear cells from the two groups of cattle was analyzed. Prior to challenge, LD cultures were established with Muguga-infected or Marikebuni 3219-infected stimulator cells. CTLp that lysed Muguga-infected target cells were detected in all of the 17 Muguga-immunized animals at frequencies ranging from 1:14,200 to 1:2,159 (Fig. 2A). When the same effector populations were assayed with target cells infected with Marikebuni 3219, CTLp were undetectable in 11 animals and were detected at frequencies ranging from 1:7,510 to 1:2,731 in 6 cattle. The six animals which exhibited cross-reactive CTLp were the same animals that were fully protected against challenge with Marikebuni 3219, as shown in Table 1. Similarly, CTLp that killed targets infected with Marikebuni 3219 were detected at frequencies ranging from 1:4,231 to 1:2,071 in all of the six animals immunized with Marikebuni 3219 (Fig. 2B). However, when tests were done with Muguga-infected target cells, although CTLp were detected at comparable frequencies ranging from 1:5,502 to 1:2,867 in four animals, they were undetectable in two of the cattle (E59 and E82). Again, those animals that had cross-reactive CTLp were the ones which were protected against challenge with *T. parva* (Muguga) (Table 2). These results demonstrate that immunization of cattle with the Muguga stock or the Marikebuni 3219 population of *T. parva* induces strain-specific CTL responses in some individuals and cross-reactive responses in others and, further, that the strain specificity of the CTLp correlates with protection against challenge with the heterologous parasite. In this study, as in previous studies (7, 23), the same cytotoxic effector cells killed target cells infected with either parasite population in cattle which generated cross-reactive CTL, although in all of these animals there was an additional small population of strain-specific CTL (data not shown), suggesting that a proportion of the effectors in these animals recognize antigenic epitopes shared between the two parasite populations.

The results of this study have established a clear relationship between the parasite strain specificity of CTL responses to *T. parva* in individual animals and their susceptibility to challenge with heterologous parasite populations. The results were consistent in animals immunized with one of two different parasite populations (one being a clone) prior to challenge with the heterologous parasite. All animals that developed cross-reactive CTL responses were protected against heterologous challenge, whereas those that had strain-specific responses were completely or partially susceptible to challenge.

Many of the animals that generated a strain-specific CTL response eventually recovered from infection following heterologous challenge. It is likely that under field conditions, when cattle would have poor nutrition and management, more of these animals would have succumbed to infection. Nevertheless, the results indicate that immunization had stimulated a partially protective response. This may have involved the generation of low frequencies of cross-reactive CTL that were below the level of detection by the assay. Alternatively, responses other than class I MHC-restricted CTL may have influenced the outcome of the infection. Thus, $CD4^+$ T-cell responses specific for parasitized cells have been demonstrated to occur in immune animals (1). Priming of $CD4^+$ T cells capable of providing help for the generation of CTLs may have allowed an accelerated priming of CTLs responding to the challenge infection, or indeed, $CD4^+$ T cells may have exerted effector functions either directly or indirectly by recruitment of accessory cells such as macrophages.

Previous studies (11) have demonstrated that immunization of cattle with the Marikebuni stock results in protection against challenge with a range of other stocks, including Muguga. It is unclear whether this property is due to the observed heterogeneity within the stock and thus a capacity to induce an immune response encompassing a range of different specificities or to the presence of a parasite component with the ability to generate immune responses to conserved antigenic determinants. Although the results of this study may favor the former interpretation, i.e., that Marikebuni 3219, unlike the parent Marikebuni stock, 3014, failed to protect all animals against challenge with the Muguga stock, it is possible that clone 3219 does not represent the component with the capacity to generate immunity to conserved epitopes. As with Muguga-immunized animals, this failure to protect against heterologous challenge correlated with the detection of strain-specific CTLp in the susceptible animals.

As far as can be determined from the analyses with parasite-specific MAb, the cloned population of the Marikebuni stock that was used in this study was indeed antigenically homogeneous and the parasites in cell lines established following tick passage were similar to those in the original cloned cell line. Although a cloned population of the Muguga stock was not used in the study, there is evidence that this stock is relatively homogeneous; while some heterogeneity at the DNA level has been detected (18a), Muguga-infected cell lines, including those used in the present study, generally exhibit a constant profile with MAb (16, 25) and are consistently recognized by Muguga-specific CTL clones of the appropriate MHC restriction specificities (8). Moreover, in a number of studies involving infection of animals with cloned Muguga-infected cell lines and subsequent challenge with the parent stock, there have been no instances of breakthrough infection (17, 18a). The greater heterogeneity within the Marikebuni stock may reflect the fact that it is a relatively recent field isolate in comparison with Muguga, which has undergone repeated passages between tick and cattle under laboratory conditions.

The finding of variation between animals in the strain specificity of CTL responses clearly indicates that individual animals respond to different antigenic epitopes and strongly suggests that this response in individuals is focussed on a limited number of epitopes. These are also characteristic features of CTL responses to viruses in mice and humans (9, 27). The question of what determines the variation in specificity of the response in individual cattle has been addressed. It is well established that in murine systems epitope selection by different class I molecules can influence the antigenic specificity of CTL responses (26). Although the results of work with twin and class I MHC haplotype-related cattle immunized against Muguga or Marikebuni 3219 indicate that the MHC phenotype plays some role in determining the strain specificity of CTLs, the immunizing parasite has been found to have a profound influence on CTL specificity (unpublished data). The parasite antigens recognized by *T. parva*-specific CTL have not yet been identified, but this is also an area that is being actively investigated. The evidence provided by the present study that CTL responses play an important role in immunity to *T. parva* clearly points to the potential of utilizing the target antigens for vaccination. Moreover, the identification of such antigens, together with the assay systems that have been developed for *T. parva*, will provide a well-defined model system to study CTL epitope selection in intracellular protozoan parasite infections.

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