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Absence of virus-induced lymphocyte suppression and interferon production in multiple sclerosis

(cell-mediated immunity/suppressor cell/viral antigens/slow neurologic diseases)

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ABSTRACT **Lymphocytes from normal adult donors exposed *in vitro* to inactivated measles virus were found to exert significant suppression (33.9%) of the concanavalin A responses of cryopreserved, autochthonous responder cells. In marked contrast, lymphocytes from multiple sclerosis patients exhibited significantly reduced suppression (1.5%), and in 80% of cases failed to suppress at all. The degree of suppression increased slightly with age of the patient but did not vary with the clinical stage of disease. There was no apparent genetic restriction of suppressor activity. Although specificity of this phenomenon for measles virus has not been established, no differences in the responses of lymphocytes from normal or multiple sclerosis patient donors were found with subacute sclerosing panencephalitis, Sendai, canine distemper, mumps, or influenza viruses.**

Supernates of measles-treated lymphocytes from normal donors possessed both suppressive and antiviral activities. Both activities were resistant to pH 2 treatment and were neutralized by an anti-human leukocyte interferon antiserum, strongly suggesting that interferon (probably type I) was the mediator of suppression. Consistent with their inability to suppress concanavalin A responses, lymphocytes from multiple sclerosis patients failed to produce significant amounts of interferon in response to measles challenge *in vitro*. These results extend previous observations that multiple sclerosis patients are unable to respond appropriately to measles virus antigen *in vitro*.

The etiology and pathogenesis of multiple sclerosis (MS), a slow neurological disease of man, remain unknown. Epidemiological and serological studies have suggested that an infectious agent might be responsible for this disease (reviewed in ref. 1). Measles virus has received perhaps the most attention as a consequence of the observation of increased measles antibodies in the sera and cerebrospinal fluids of MS patients (2) and the isolation of a measles-like virus from the brain and lymph nodes of patients with another slow neurological disease, subacute sclerosing panencephalitis (SSPE) (3, 4). Histological examination of brains from MS patients frequently reveals perivascular infiltration by hematogenous cells (5), suggesting an immunological mechanism for the demyelination. Many investigators have studied the *in vitro* cell-mediated immune responses of MS patients in an attempt to demonstrate aberrant responses to specific antigens, particularly to viral antigens (6–8). Of particular interest, lymphocytes from MS patients have been reported to show decreased leukocyte migration inhibition to measles antigen (9, 10) and increased rosette formation with human epithelial cells persistently infected with measles virus (11).

In the present study, we have investigated the *in vitro* antigen-specific induction of suppressor activity in lymphocytes from normal adult and MS patient donors, using measles virus. Our results show that, in response to measles challenge, lymphocytes from MS patients exhibit an almost total absence of suppressor activity. In addition, they fail to produce significant amounts of a soluble immunosuppressive and antiviral factor which appears to be interferon.

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MATERIALS AND METHODS

Lymphocyte Donors. Lymphocytes were separated, by Ficoll-Paque (Pharmacia) density gradient centrifugation, from 50 ml of heparinized blood drawn from the following groups of donors: normal adults (mean age, 35 yr; laboratory personnel); MS patients (mean age, 40 yr) who were diagnosed by a board-certified neurologist as having "clinically definite" MS according to the criteria of Schumacher *et al.* (12); patients with cerebrovascular disease (strokes) (mean age, 63 yr), all of whom were middle-aged or elderly, presented with the acute onset of neurological deficit, and had a diagnosis of ischemic infarction supported by clinical and neuroradiological evaluation; children (mean age, 1 yr) who had not been vaccinated against measles and who were found to be measles seronegative by virus neutralization assay. All adult donors (i.e., normal, MS, and stroke) were measles seropositive by virus neutralization assay. None of the MS patients was currently receiving steroids or corticotropin treatment.

Antigens. The Edmonston strain of measles virus (referred to as measles virus), the Mantooth strain (13) of SSPE virus (SSPE virus), Sendai virus, and canine distemper virus (CDV) were prepared by infecting Vero cell monolayers at multiplicities of infection of 0.01. The supernates were harvested at the time of maximal cytopathic effect, centrifuged at 1000 × *g* to remove cell debris, and inactivated with β-propiolactone (0.15%, 37°C, 2 hr) (14). Complete inactivation was demonstrated by the absence of detectable cytopathic effect on Vero indicator cells. The virus concentration was adjusted prior to inactivation to give the equivalent of 0.1 plaque-forming or infectious unit per lymphocyte during modulator treatment. Mumps and influenza type A were hemagglutination and complement-fixation antigen preparations, respectively, were obtained from Microbiological Associates (Bethesda, MD), and were used undiluted after β-propiolactone inactivation. Control antigen was the supernate from uninfected Vero cells prepared as described above.

Induction of Suppression. Lymphocytes from each donor (4×10^6 cells per 0.2 ml) were incubated for 2 hr in 5-ml plastic tubes with 0.2 ml of control or viral antigen. All lymphocyte manipulations were performed in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. After 2 hr at 37°C, the lymphocytes were washed twice and suspended in 2 ml of fresh medium. An additional 4×10^6 lymphocytes from each donor were cultured with 40 μg of concanavalin A (Con A; Miles) in 2 ml of medium. After culture for 72 hr at 37°C in 5% CO_2 in air, the antigen- and Con A-treated cells (*modulators*)

Abbreviations: MS, multiple sclerosis; SSPE, subacute sclerosing panencephalitis; CDV, canine distemper virus; Con A, concanavalin A.

were separated from their culture medium (*supernates*) by centrifugation. The modulators were washed thoroughly, inactivated with mitomycin C (50 μg/ml, 37°C, 30 min), washed three times, and counted. The optimal antigen concentration, duration of lymphocyte treatment, and modulator/responder ratio were determined by preliminary experiments.

Assay for Suppression. Lymphocytes from each donor were cryopreserved in 10% dimethyl sulfoxide in RPMI 1640 containing 20% fetal calf serum and antibiotics and stored under liquid N_2 until required. They were thawed 3 days later, washed, and counted to provide *responder* cells. Mixed cultures of autochthonous responder cells (2×10^5) and either modulator cells ($1\text{–}2 \times 10^5$) or supernates (0.1 ml) were prepared in the wells of flat-bottomed microtiter plates in a total volume of 0.2 ml and cultured for 3 days alone or with 2 μg of Con A per well. At 16 hr prior to cell harvest, 1 μCi of [^{3}H]thymidine (Amersham/Searle; specific activity, 3 Ci/mmol) was added to each well. Cultures were harvested on glass fiber filters with a Skatron semi-automatic cell harvester and assayed for radioactivity in a Beckman liquid scintillation counter. All determinations were done in triplicate. The percentage suppression was calculated as

$$100 \times 1 - \left[\frac{\text{Con A response of responders + treated modulators or supernates}}{\text{Con A response of responders + control modulators or supernates}} \right]$$

and expressed as the mean (±SEM) for each donor group. Con A responses in cpm were statistically analyzed by Student's *t* test; percentage suppression values were analyzed by the Wilcoxon rank sum test. Significance was accepted at the $P < 0.05$ level.

Assay and Characterization of Antiviral Activity. Serial 0.5 $\log_{10}$ dilutions of each supernate were prepared, and 0.1 ml of each dilution was applied to a confluent monolayer of human trisomic-21 skin fibroblasts (GM 2504; Institute for Medical Research, Camden NJ) in microtiter wells. After 18 hr, the supernates were removed, and 0.2 ml of vesicular stomatitis virus (Indiana strain) containing 5×10^4 plaque-forming units was added to each well. The antiviral activity was determined 48 hr later as the reciprocal of the highest dilution that inhibited 50% of the viral cytopathic effect. Each assay included a reference interferon preparation (National Institutes of Health human reference interferon, G-023-901-527) for standardization, and 1.5 antiviral units was found to be equivalent to 1 reference unit of NIH interferon.

For pH 2 treatment, supernates were dialyzed for 48 hr against RPMI 1640 medium adjusted to pH 2 with HCl and then for 48 hr against RPMI 1640 medium alone (pH 7.5). Untreated supernates were dialyzed in parallel for 96 hr against RPMI 1640 at pH 7.5.

Rabbit anti-human leukocyte interferon globulin was prepared by K. Paucker and kindly provided by G. Galasso (National Institutes of Health). This antiserum had an antibody titer of 10^4 units/ml against human leukocyte interferon and 320 units/ml against human fibroblast interferon. The antiserum was incubated with the supernates at a final dilution of 1:80 for 30 min at 37°C prior to assay for suppression or antiviral activity.

RESULTS

Suppression of Measles-Treated Modulator Cells. Peripheral blood lymphocytes from normal adults and from patients with various stages of MS were treated with measles virus or Con A and cultured for 3 days. After mitomycin C treatment, they were tested for their ability to suppress the Con A mitogenic responses of autochthonous responder cells in mixed cultures. Whereas such measles-treated modulators from normal donors exerted a highly significant suppression of the Con A response, those from MS patients almost totally failed to suppress (Table 1). Eighty percent (29/36) of the MS patients tested failed to exhibit any significant suppression, in contrast to only 12% (4/32) of the normal donors. Although both infectious and β-propiolactone-inactivated measles were equally suppressive, inactivated virus was chosen for use in the present study to eliminate the potential complications of lymphocyte infection, because Lucas *et al.* (15) recently reported that live measles virus inhibits mitogen responses by actual infection of the lymphocyte. Con A induced significant suppression of [^{3}H]thymidine incorporation in both donor groups, but MS patients again exhibited markedly less suppression (Table 1). These data also indicate that the reduced suppression by both measles and Con A modulators from MS patients was not due to impaired Con A responses of responder cells from these patients.

Modulators that suppressed autochthonous responder cells were also found capable of suppressing allogeneic cells as shown in a representative experiment (Table 2). In addition, modulators from normal donors suppressed both normal and patient responder cells but those from patients failed to suppress either. These results show that the suppressor activity was probably not genetically restricted; the absence of suppression in MS patients resulted from the failure of modulators from these patients to be suppressive rather than an inability of their responder cells to be suppressed.

Variation in Suppression with Age. There have been conflicting reports concerning the variation in Con A suppressor activity with age (16, 17). For this reason, the data from these experiments were also grouped by age, and the percentage suppression observed in MS patients under and over age 50 yr was compared with that of appropriate age-matched normal donors. Although measles-induced suppression increased with

Table 1. Measles- and Con A-induced suppression in normal and MS patient donors

Modulator treatment	Normal (n = 32) Con A response,* cpm	Normal (n = 32) Suppression,† %	MS patient (n = 36) Con A response,* cpm	MS patient (n = 36) Suppression,† %
Control	46,084 ± 6,502	—	63,204 ± 9,207‡	—
Measles	30,448 ± 4,637§	33.9	62,227 ± 9,235	1.5
Con A	20,946 ± 4,298§	54.5	39,289 ± 6,295§	37.8

* Calculated as the arithmetic mean (±SEM) cpm of cultures with Con A after subtraction of the mean cpm of cultures without Con A.

† Percentage reduction in the mean Con A response of mixed cultures with treated modulators compared to the mean response of mixed cultures with control modulators.

‡ No significant difference from the Con A response of normal donors ($P > 0.05$, Student's *t* test).

§ Significantly different from Con A response of mixed cultures containing control modulators ($P < 0.001$, paired-sample Student's *t* test, each donor serving as his own control).

Table 2. Measles-induced suppression of autochthonous and allogeneic responder cells

Donor cells		Con A response,* cpm		Suppression,
Modulator	Responder	Control	Measles	%
B.B. (normal)	B.B. (normal)	71,013	43,680	38.5
	W.S. (normal)	66,107	43,237	34.6
	A.N. (normal)	58,375	38,912	33.3
	S.U. (MS)	57,623	37,350	35.2
S.U. (MS)	S.U. (MS)	56,267	55,269	1.8
	W.S. (normal)	55,905	53,223	4.8
	A.N. (normal)	94,525	88,822	6.0
	D.B. (MS)	60,585	60,591	0.0

* Con A response of responder cells plus either control- or measles-treated modulators.

age in MS patients, it remained significantly lower than that observed in age-matched controls (Fig. 1*a*). Interestingly, the normal donors did not exhibit increased measles-induced suppression with increasing age.

Variation in Suppression with Clinical Stage of Disease. Antel and Arnason (18, 19) have reported variations in Con A-induced suppression with clinical stages of MS. In the present study, analysis of data from 26 MS patients (<50 yr old) showed no significant differences ($P > 0.05$) in the amount of measles-induced suppression between patients undergoing the acute phase of an exacerbating course (2.2 ± 5.2%, $n = 9$), stable patients (9.0 ± 10.0%, $n = 5$), or chronic progressive patients (6.3 ± 4.6%, $n = 12$). However, Con A-induced suppression varied significantly in a manner consistent with Antel and Arnason's findings: i.e., acute, 22.7 ± 12.6%; stable, 60.0 ± 12.3%; and chronic progressive, 40.3 ± 5.4%.

Suppression in Non-MS Donors. In order to determine whether the observed loss of suppressor activity was restricted to MS or was a feature of other neurological disorders, patients with cerebrovascular disease (strokes) were tested for measles- and Con A-induced suppression. There were no significant differences in the mean percentage suppression induced by measles or Con A in these patients compared with age-matched normal donors (Fig. 1). Non-measles-vaccinated seronegative children were also tested for suppression. Their measles-treated modulators failed to exert any significant suppression, whereas

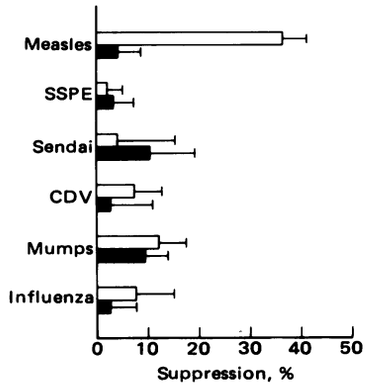

FIG. 2. Suppression of the Con A mitogenic responses of responder lymphocytes by modulators from 12 normal adults (open bars) and 12 MS patients (solid bars) treated with various viral antigens. Mean percentage suppression induced by measles in MS patients was significantly lower ($P < 0.001$, Wilcoxon rank sum test) than in normal donors. No other significant difference ($P > 0.05$) was observed with any of the other viruses.

the level of Con A-induced suppression was similar to that observed in normal adult donors (Fig. 1).

Specificity of Suppressor Induction. Measles and several other related and unrelated viruses were tested in this experimental system to determine whether the inability of MS patients to suppress was a measles-specific phenomenon. Six viruses were examined: measles, SSPE virus, Sendai, CDV, mumps, and influenza type A. Of these, only measles virus consistently suppressed the Con A responses of normal donors, and none of the viruses induced any significant suppression of MS patient responder cells (Fig. 2).

Interferon as the Mediator of Suppression. Treatment of lymphocytes from normal donors with measles virus resulted in the release of a factor(s) into the culture supernates that suppressed the Con A mitogenic responses of autochthonous responder cells (Fig. 3*a*). Supernates of measles-treated lymphocytes from MS patients, however, were significantly less immunosuppressive. As was found with the modulator cells, suppressive supernates showed no genetic restriction or specificity in their suppressive activity.

Interferons, characterized by their antiviral properties, have been demonstrated to be immunosuppressive both *in vivo* and

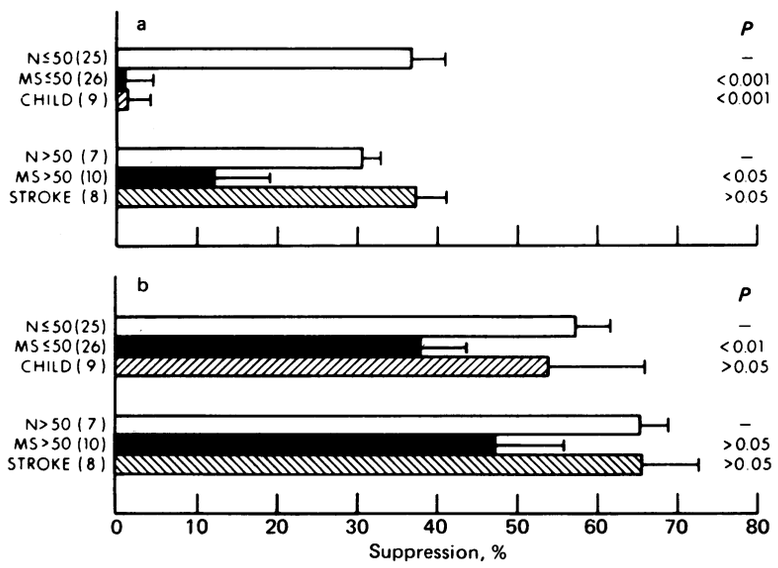

FIG. 1. Measles-induced (*a*) and Con A-induced (*b*) suppression of the Con A mitogenic responses of lymphocytes from normal adults (N); MS patients; children; and stroke patients; of various ages. Numbers in parentheses indicate group sizes. For *P*, each group was compared with an appropriate age-matched normal donor group (Wilcoxon rank sum test).

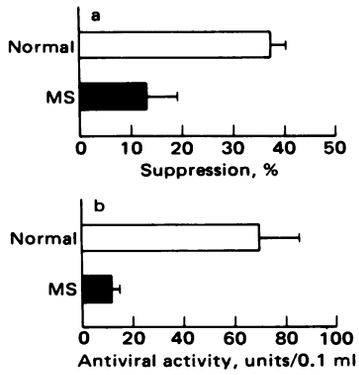

FIG. 3. Suppressive (*a*) and antiviral (*b*) activities of supernates from measles-treated lymphocytes obtained from 24 normal adults (open bars) and 24 MS patients (solid bars). Mean percentage suppression and mean antiviral activity of supernates from MS patients were significantly different ($P < 0.001$, Wilcoxon rank sum test) from those of normal donors.

in vitro (20–24). For this reason, supernates of measles-treated lymphocytes from both donor groups were assayed for antiviral activity. Supernates from normal donors were found to contain moderate levels of antiviral activity, but those from MS patients exhibited significantly lower levels (Fig. 3*b*). Supernates of control-treated lymphocytes from normal and MS patient donors did not contain any detectable antiviral activity.

Several species of human interferons have been described, and of these, two are produced by lymphoid cells in response to virus induction (type I interferon) and antigenic or mitogenic stimulation (type II interferon) (see ref. 25 for review). These interferons are antigenically dissimilar and exhibit different physical properties (26, 27). Therefore, attempts were made to identify the nature of the factors responsible for the immunosuppressive and antiviral properties of supernates from measles-treated lymphocytes. Both the suppressive and antiviral activities were found to be resistant to pH 2 treatment for 48 hr, whereas both activities were neutralized by an anti-human leukocyte interferon globulin. A representative experiment is shown in Table 3. Simultaneous pH 2 treatment of a supernate from Con A-stimulated lymphocytes, known to contain type II interferon (25), reduced its suppressive activity from 21.8% to 0.2% and its antiviral titer from 32 units/ml to <1 unit/ml. This result indicates that the conditions used for pH 2 treatment were sufficient for complete inactivation of any type II interferon present. To determine the role of interferon in modulator-mediated suppression, anti-interferon serum was added to mixed cultures of measles-treated modulators and responders. In a representative experiment, the anti-interferon serum reduced the measles-induced suppression from 65.9 to 37.7%.

DISCUSSION

The present studies support previous observations of abnormal responses of MS patients' lymphocytes to measles virus *in vitro*. Measles-treated lymphocytes from 28 of 32 normal donors were found to exert reproducible and significant suppression of the Con A responses of cryopreserved responder lymphocytes. In contrast to reports of some other lymphocyte-mediated suppressor systems (28–30), there appeared to be no genetic restriction of the suppressive activity of measles-treated modulator cells because suppressive modulators suppressed both autochthonous and allogeneic responder cells. Lymphocytes from 36 MS patients exhibited significantly reduced suppression, and lymphocytes from 29 of these patients failed to suppress the Con A responses at all. This failure was not due to the inability of patients' responder cells to be suppressed. Rather, the defect lies in the inability of the measles-treated modulators to exert suppression.

Table 3. Characterization of suppressive and antiviral activities of a supernate from lymphocytes exposed to measles virus

Supernate treatment	Suppression, %	Antiviral activity, units/ml
Untreated	41.1	317
pH 2	41.6	317
Anti-interferon globulin	6.1	<1

Measles-induced suppression was found to increase slightly with age in MS patients but not in normal donors. This might reflect possible changes in immune function associated with progression of the disease rather than with aging. In contrast to the Con A suppressor system (18, 19), measles-induced suppression did not appear to vary with the clinical stage of disease. Patients with cerebrovascular disease appeared to suppress normally in response to measles and Con A. Although this result suggests some specificity of the phenomenon for MS, other neurological diseases such as amyotrophic lateral sclerosis and myasthenia gravis have yet to be examined. Some initial studies with patients suffering from rheumatoid arthritis indicate normal levels of measles-induced suppression.

Lymphocytes from measles seronegative donors failed to suppress in response to measles virus despite normal Con A suppressor activity. Possible explanations for this might be that antigenic recognition resulting from prior exposure to measles is an essential requirement for measles-induced suppression or that the mechanism of measles-induced suppression is nonfunctional at this early age. It is interesting that the SSPE virus, which is a variant of measles virus (3, 4), failed to induce any suppression. This suggests that the recognition of determinants peculiar to measles virus is required for the induction of this suppressor activity. Another paramyxovirus, Sendai virus, also failed to suppress lymphocytes from normal and MS patient donors. This virus shows a close serological and structural homology to parainfluenza 6/94 virus which was isolated from brain cells of a MS patient (31). CDV, which produces a demyelinating encephalitis in dogs (5) and has recently been associated epidemiologically with MS (32), did not induce any significant suppression. Of the six viruses tested, measles was the only viral antigen that induced significant suppression in normal lymphocytes. Additional experiments have indicated that the suppressive activity of measles-treated modulators is abrogated by an anti-measles serum (data not shown), and it is known that measles virus binds to human lymphocytes (33). These observations suggest that the transfer of measles is required for suppression of the responder cells. For these reasons, it cannot be concluded that the failure of measles virus to suppress MS lymphocytes was specific for this antigen.

Exposure of lymphocytes from normal donors to measles resulted in the release of a factor(s) with immunosuppressive and antiviral properties. In marked contrast, however, supernates from MS patients' lymphocytes appeared to exhibit significantly less of these activities. Further characterization of these activities showed them both to be resistant to pH 2 and to be neutralized by an anti-human leukocyte type I interferon serum. This evidence strongly suggests that the antiviral activity is caused by type I leukocyte interferon, produced in response to measles challenge *in vitro*. In addition, the suppressive activity of the supernates was resistant to pH 2, and the suppressive activities of both supernates and modulators were neutralized by an anti-human leukocyte interferon serum. Therefore, we conclude that type I human leukocyte interferon is the likely mediator of measles-induced suppression in this system.

Interferons can be produced in response to various inducers by T and B cells (34), macrophages (35), and natural killer (NK)

cells (36). The nature of the cell responsible for suppression and interferon production in this system remains unknown. Preliminary experiments (to be reported elsewhere) have shown that measles-induced suppression is probably mediated by a lymphocyte that does not adhere to nylon wool or form rosettes with sheep erythrocytes, indicative of non-T, non-B lymphocytes. It has been reported recently that MS patients have an increased proportion of a specific T-cell subset in their circulating lymphocytes (37). This subset of cells is characterized by Fc receptors for IgG immune complexes (Tγ cells) and is believed to contain T suppressor cells (38). However, the functional activity of these cells in MS patients is not known, and the relevance of this finding to the present study is unclear.

The apparent inability of MS patients to produce interferon in response to virus challenge suggests some interesting possibilities that might be relevant to the pathogenesis of this disease. Clearly, interferons possess antiviral activity, and the inability of lymphocytes from MS patients to produce these factors could contribute to the persistence of virus in the central nervous system. More intriguing, however, are recent observations showing that natural killer cells, which are non-T, non-B lymphocytes responsible for spontaneous cytotoxicity against tumor cells (39), play an important role in the cytolysis of virus-infected cells (40, 41). In addition, administration of interferon or interferon inducers *in vivo* and *in vitro* enhances the cytolytic activity of natural killer cells (42, 43). Therefore, it is possible that the failure of lymphocytes from MS patients to "recognize" virus antigens and produce interferon might lead to the impairment of natural killer cell regulation of intracellular virus infections. Although this hypothesis requires further experimental investigation, the results of the present study show that lymphocytes from MS patients are unable to respond appropriately to measles virus antigens.

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