

THE IMMUNOLOGICALLY SPECIFIC RETENTION OF  
RECIRCULATING LONG-LIVED LYMPHOCYTES IN  
LYMPH NODES STIMULATED BY  
XENOGENEIC ERYTHROCYTES\*

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We have previously demonstrated that lymph nodes draining the site of an allograft reaction selectively retain long-lived lymphoid cells, in part on the basis of the immunological specificity of the cells involved (1). The relatively small immunologically specific component in this accumulation was demonstrated by a double isotope dilution method, in which cells sensitized to one set of histocompatibility antigens were labeled *in vivo* with thymidine-<sup>3</sup>H and cells sensitized to a different set of histocompatibility antigens were labeled with thymidine-<sup>14</sup>C, under conditions where most of the label was confined to the long-lived cells (1-3). The cells were then pooled and adoptively transferred to syngeneic hosts who were then challenged with both sets of antigens. Immunological specificity was inferred from the small but consistent excess of cells from the donors immunized to each set of antigens in the lymph nodes stimulated by that same antigen 6-8 days after challenge grafting. In these initial studies (1) we deliberately selected an immunological reaction that was principally dependent on cell-mediated immunity (an allograft reaction in *H-2*-compatible strains). The present studies were designed to ascertain whether a similar selective accumulation of long-lived lymphocytes occurs in lymph nodes stimulated by antigens evoking principally an immunoglobulin-mediated response, and to demonstrate a similar immunologically specific component in this accumulation.

*Materials and Methods*

*Animals.*—Genetically inbred female CBA/J mice and male and female A/Jax mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

*Immunization.*—Groups of CBA/J mice, not less than 8 wk of age, were immunized to either sheep red blood cells (SRBC)<sup>1</sup> (Grand Island Biological Co., Grand Island, N.Y.) or chicken RBC (CRBC) (courtesy of Doctors L. W. Schierman and R. A. McBride) by a single

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<sup>1</sup> *Abbreviations used in this paper:* CRBC, chicken red blood cells; dpm, disintegrations per minute; SRBC, sheep red blood cells.

intravenous injection of 0.20 ml of a 20% suspension of thrice-washed cells and a simultaneous intracutaneous injection of 0.1 ml of a similar 40% suspension divided among the four foot-pads.

In the preliminary experiments similar groups of A/Jax mice were immunized by intravenous injection alone (0.20 ml of a 20% suspension). In one experiment (results summarized in Table II) a single injection of the second antigen was given 10 days later, after the course of thymidine-<sup>3</sup>H. (These animals were originally prepared as the control groups for an experiment designed to demonstrate immunological specificity using only one radioisotope.)

*Labeling of Long-Lived Lymphoid Cells.*—Long-lived lymphoid cells were labeled as previously described (1). In brief, donor mice were given intraperitoneal injections of thymidine-<sup>3</sup>H (total dose 2–18  $\mu$ Ci/g) or thymidine-<sup>14</sup>C (total dose 2–4  $\mu$ Ci/g) (New England Nuclear Corp., Boston, Mass.) twice daily for 5–12 days commencing 48 hr after immunization. The donor mice were then rested for at least 3 wk after the last injection of isotope to permit the short-lived lymphoid cells to dilute out their label.

*Preparation of Cells for Adoptive Transfer.*—Spleen and lymph nodes (cervical, axillary, brachial, iliac, inguinal, popliteal, and mesenteric) were minced in Hanks' balanced salt solution and passed through a series of graded stainless steel screens as previously described (1).

*Challenge.*—CBA/J mice were challenged intracutaneously with 0.05 ml of a 40% suspension of CRBC divided between the right front and rear paws, and similarly with 0.05 ml of 40% SRBC in the left front and rear paws. In preliminary experiments A/Jax mice were challenged in various ways in the right front and rear paws only. Syngeneic RBC for challenge were obtained from the orbital plexuses of A/Jax mice and washed three times in saline. Colloidal carbon (Pelikan C11/1431a, Günther Wagner Co., Hanover, Germany) and turpentine were used for nonimmunological challenge.

*Experimental Design.*—In preliminary experiments designed to define the rate of accumulation of long-lived cells in antigenically stimulated lymph nodes and to define some of the non-specific stimuli that can cause cellular retention, A/Jax mice were immunized as described above and given a 5–12 day course of thymidine-<sup>3</sup>H. 3 wk after the last injection of thymidine-<sup>3</sup>H the animals were challenged and sacrificed at various times thereafter; the lymph nodes draining the challenged paws were compared radiochemically with those draining the non-challenged paws.

In the double-label experiments directly concerned with immunological specificity, groups of CBA/J mice were immunized to either CRBC or SRBC and given a 12 day course of either thymidine-<sup>3</sup>H or thymidine-<sup>14</sup>C starting 48 hr after immunization. 3 wk after the last injection of isotope the animals were sacrificed and the lymph node and spleen cells of the two groups were pooled and transferred (intravenously) to syngeneic recipients. In the experiments shown in Table III and V the animals immunized to CRBC were given thymidine-<sup>3</sup>H and those immunized to SRBC were given thymidine-<sup>14</sup>C. In the experiment shown in Table IV the radioactive labels were reversed: animals immunized to CRBC received thymidine-<sup>14</sup>C and those immunized to SRBC received thymidine-<sup>3</sup>H. Recipients were challenged as described above either 3 days before, at the same time as, or 1 day after the adoptive transfer. The first and second of these groups were sacrificed 24 hr after adoptive transfer. The third group was sacrificed 24 hr after antigenic challenge. Draining (popliteal, iliac, axillary, and brachial) and nondraining (cervical and inguinal) lymph nodes were removed and segregated according to side for radiochemical analysis.

*Preparation of Samples and Counting Procedures.*—Lymph nodes were dried and defatted with acetone, dissolved in N NaOH, and suspended in a dioxane Cab-O-Sil (Packard Instrument Company, Downers Grove, Ill.) cocktail as previously described (1). <sup>3</sup>H and <sup>14</sup>C were counted simultaneously (4) in a Packard Tri-Carb liquid scintillation spectrometer (model 3375) (Packard Instrument Company).

*Treatment of Data.*—The disintegrations per minute (dpm) of <sup>3</sup>H were normalized to a unit quantity of <sup>14</sup>C by taking the ratio of dpm-<sup>3</sup>H/dpm-<sup>14</sup>C. This permitted a direct comparison

of the two sets of draining lymph nodes of each recipient by dividing the normalized  $^3\text{H}$  of one side by the normalized  $^3\text{H}$  of the other. In the absence of immunologic specificity, the normalized  $^3\text{H}$  of the two sides should be equal and dividing one by the other should give a quotient of 1.00. In the presence of immunologic specificity, the normalized  $^3\text{H}$  of the two sides should differ and dividing one by the other should give a quotient greater or less than 1.00. In the present experiments these quotients were calculated so that values greater than 1.00 indicate immunologic specificity.

*Statistical Methods.*—We began these experiments with the hypothesis that lymphoid cells from donors immunized to a given antigen will preferentially accumulate in a lymph node stimulated by that same antigen. If this hypothesis is correct the normalized  $^3\text{H}$  of side A will be greater than that of side B in the experiments shown in Tables III and V and the normalized  $^3\text{H}$  of side B will be greater than that of side A in the experiment shown in Table IV. We have tested this hypothesis by two methods: the Student's  $t$  test, which is parametric, and

TABLE I  
*Accumulation of  $^3\text{H}$  in Specifically Challenged Lymph Nodes (A) as Compared to Nonchallenged Lymph Nodes (B), at Various Times during a Secondary Immune Response*

No. of animals	Sensitization*	Challenge (Footpad)	Interval after challenge <i>hr</i>	Mean ratios of cpm challenged side to control side (A/B) $\pm$ SE of mean
2	CRBC	None	—	1.00 $\pm$ 0.00
4	CRBC	CRBC	2	1.11 $\pm$ 0.06
4	CRBC	CRBC	4	1.24 $\pm$ 0.21
7	CRBC	CRBC	8	1.36 $\pm$ 0.07
13	CRBC	CRBC	24	1.83 $\pm$ 0.09
7	CRBC	CRBC	48	2.15 $\pm$ 0.12
2	CRBC	CRBC	72	1.88 $\pm$ 0.36

\* Animals were given 0.25  $\mu\text{Ci/g}$  thymidine- $^3\text{H}$  twice daily on days 2–7 after sensitization.

the sign test, which is nonparametric. In the  $t$  test, the stimulated and nonstimulated nodes of each group were compared as independent samples. The standard deviations of the stimulated and nonstimulated nodes were pooled for calculating  $t$  (5). In the sign test, the normalized  $^3\text{H}$  of A and B of each animal was compared. When A/B (Tables III and V) or B/A (Table IV) was greater than 1.00, the animal was given a plus sign. When the ratio was less than one, it was given a minus sign. The probability of obtaining the observed number of plus signs or more than the observed number of plus signs was determined from the binomial distribution (6) with  $P = 0.5$  in the expression  $\binom{n}{r} P^r q^{n-r}$  (7).

## RESULTS

The results of the first preliminary experiment defining the rate of accumulation of long-lived lymphoid cells in lymph nodes undergoing a secondary response to CRBC are summarized in Table I. There is a statistically significant accumulation of labeled cells in the stimulated nodes within 8 hr of the time of antigen administration ( $P < 0.01$ ). This accumulation approaches a plateau within 24–48 hr and does not seem to increase beyond this point. Additional experiments summarized in Table II have shown that nonimmunological in-

flammation (turpentine induced) can also cause a statistically significant retention of long-lived cells in the draining lymph nodes ( $P < 0.01$ ), but that activation of the phagocytic cells in the node by colloidal carbon does not. Syngeneic RBC also seem to have little or no effect on the retention of cells.

The experiments concerned directly with immunological specificity are sum-

TABLE II  
*Accumulation of  $^3\text{H}$  in Lymph Nodes after Specific and Nonspecific Challenge (A) as Compared to Nonchallenged (B) Nodes*

No. of animals	Sensitization*	Challenge	Interval after challenge	Mean ratios of cpm challenged side to control side (A/B) $\pm$ SE of mean
			<i>hr</i>	
8	CRBC and SRBC	Mouse RBC (syngeneic)	24	1.11 $\pm$ 0.05
8	CRBC and SRBC	Turpentine	24	1.63 $\pm$ 0.10
7	CRBC and SRBC	Colloidal carbon	24	1.18 $\pm$ 0.17

\* Animals were immunized with CRBC or SRBC on day 0, given 0.25  $\mu\text{Ci/g}$  thymidine- $^3\text{H}$  twice daily on days 2-7, then given the other antigen (CRBC or SRBC) on day 12. Challenge was on day 38 or 45.

TABLE III  
*Accumulation of Passively Transferred Anti-Chicken RBC Lymphoid Cells Labeled with Thymidine- $^3\text{H}$  and Anti-Sheep RBC Cells Labeled with Thymidine- $^{14}\text{C}$  in Lymph Nodes Stimulated with Chicken RBC or Sheep RBC, and in Nonstimulated Control Lymph Nodes*

Recipient No.*	Stimulated nodes			Nonstimulated nodes		
	Normalized $^3\text{H}\ddagger$ in lymph nodes stimulated with			Normalized $^3\text{H}\ddagger$ in lymph nodes homolateral to those stimulated with		
	Chick RBC (A)	Sheep RBC (B)	A/B§	Chick RBC	Sheep RBC	A/B§
1	2.95	2.69	1.10	2.76	2.74	1.00
2	2.87	2.65	1.08	2.69	2.77	.97
3	2.96	2.66	1.11	2.78	2.73	1.02
4	2.83	2.62	1.08	2.70	2.71	1.00
5	2.89	2.66	1.09	2.73	2.78	.98
6	2.93	2.74	1.07	2.80	2.93	.96
7	5.06	4.53	1.12	5.04	4.73	1.07
8	5.15	4.62	1.12	4.80	4.90	.98
		Mean	1.10		Mean	1.00

\* Animals 1-6 received pooled spleen cells and peripheral lymph node cells of six mice immunized to chick RBC and six mice immunized to SRBC. Animals 7 and 8 received the pooled mesenteric lymph node cells of the same 12 donors.

$\ddagger$  dpm  $^3\text{H}$  relative to dpm  $^{14}\text{C}$  ( $^3\text{H}/^{14}\text{C}$ ).

$\S$  Ratio greater than 1.00 indicates immunological specificity.

marized in Tables III–VI. Table III shows the accumulation of thymidine- $^3\text{H}$ -labeled long-lived lymphoid cells from CBA/J donors immunized to CRBC and thymidine- $^{14}\text{C}$ -labeled cells from donors immunized to SRBC in the lymph nodes of recipients challenged with both antigens. In all eight animals the lymph nodes stimulated with CRBC have retained a small excess of long-lived cells from the donors immunized to CRBC and the nodes stimulated with SRBC have retained a small excess of cells from donors immunized to SRBC. The mean value of the normalized  $^3\text{H}$  in lymph nodes stimulated with CRBC relative to the normalized  $^3\text{H}$  in lymph nodes stimulated with SRBC (A/B)

TABLE IV  
*Accumulation of Passively Transferred Anti-Chicken RBC Lymphoid Cells Labeled with Thymidine- $^{14}\text{C}$  and Anti-Sheep RBC Cells Labeled with Thymidine- $^3\text{H}$  in Lymph Nodes Stimulated with Chicken RBC or Sheep RBC, and in Nonstimulated Control Lymph Nodes*

Recipient No.*	Stimulated nodes			Nonstimulated nodes		
	Normalized $^3\text{H}\ddagger$ lymph nodes stimulated with			Normalized $^3\text{H}\ddagger$ lymph nodes homolateral to those stimulated with		
	Chick RBC (A)	Sheep RBC (B)	B/A§	Chick RBC (A)	Sheep RBC (B)	B/A§
9	1.76	1.92	1.09	1.91	1.99	1.04
10	1.78	2.03	1.15	2.17	2.14	.99
11	1.68	2.00	1.19	2.03	2.03	1.00
12	1.74	2.02	1.16	2.13	2.12	1.00
13	1.70	1.96	1.15	2.03	2.08	1.03
14	1.67	1.91	1.14	1.93	2.00	1.04
15	2.51	3.00	1.20	3.18	3.19	1.00
16	2.78	2.93	1.05	3.13	2.96	.95
		Mean	1.14		Mean	1.01

\* Animals 9–14 received pooled spleen cells and peripheral lymph node cells of six mice immunized to chick RBC and six mice immunized to SRBC. Animals 15 and 16 received the pooled mesenteric lymph node cells of the same 12 donors.

$\ddagger$  dpm  $^3\text{H}$  relative to dpm  $^{14}\text{C}$  ( $^3\text{H}/^{14}\text{C}$ ).

§ Ratio greater than 1.00 indicates immunological specificity.

equals 1.10, while the nonstimulated control lymph nodes behave as theory would predict, with a mean of 1.00. Table IV shows the results of a second experiment, identical in every way except that the radioactive labels given to the donors were reversed. Once again eight out of eight sets of stimulated nodes have shown immunological specificity in the long-lived donor cells they have selectively retained (mean of B/A = 1.14), and the control nondraining nodes have shown the expected small random variations (mean = 1.01). Table V summarizes the results of an experiment exploring the effects of varying the relationship between the times of antigenic challenge and adoptive immunization. Group A was challenged 3 days before adoptive immunization,

group B was challenged immediately before adoptive transfer, and group C was challenged 1 day after transfer. In all groups, five out of five animals showed immunological specificity in the distribution of the donor cells. There were no significant differences in the size of the immunologically specific component among the three groups.

The results of the three experiments summarized in Tables III-V have been analyzed statistically, and the differences in the stimulated nodes have

TABLE V  
*Immunologically Specific Retention of Long-Lived Lymphoid Cells In Adoptively Immunized Mice Challenged with Chicken and Sheep RBC at Various Times*

Group	No. of animals	Day of challenge*	Day of sacrifice*	Mean of A/B values for stimulated nodes $\pm$ SE of the mean	Mean of A/B values for pairs of control nodes $\pm$ SE of the mean
A	5	-3	+1	1.08 $\pm$ 0.01	1.02 $\pm$ 0.01
B	5	0	+1	1.06 $\pm$ 0.01	0.99 $\pm$ 0.01
C	5	+1	+2	1.06 $\pm$ 0.01	0.98 $\pm$ 0.01

\* Relative to adoptive transfer of labeled immunized cells on day 0.

TABLE VI  
*Summary of the Statistical Analyses of Experiments Shown in Tables III-V (Experiments 3-5)*

Experiment No.	No. of mice	Student's <i>t</i> test				Sign test					
		Mean A/B or B/A $\pm$ SE of mean, stimulated nodes	Mean A/B or B/A $\pm$ SE of mean, control nodes	<i>t</i>	<i>P</i> *	Stimulated nodes			Control nodes		
						+	-	<i>P</i> *	+	-	<i>P</i> *
3	8	1.10 $\pm$ 0.01	1.00 $\pm$ 0.01	7.14	<0.01	8	0	<0.01	2	4	N.S.‡
4	8	1.14 $\pm$ 0.02	1.01 $\pm$ 0.01	5.90	<0.01	8	0	<0.01	3	2	N.S.
5A	5	1.08 $\pm$ 0.01	1.02 $\pm$ 0.01	4.86	<0.01	5	0	<0.05	3	1	N.S.
5B	5	1.06 $\pm$ 0.01	0.99 $\pm$ 0.01	4.42	<0.01	5	0	<0.05	3	1	N.S.
5C	5	1.06 $\pm$ 0.01	0.98 $\pm$ 0.01	5.86	<0.01	5	0	<0.05	1	4	N.S.
Total	31	1.09 $\pm$ 0.01	1.00 $\pm$ 0.01	—	—	31	0	<0.001	12	12	N.S.

\* Probability that the observed differences are due to chance.

‡ Not significant.

been found to be significant. The results of these analyses are summarized in Table VI.

In all, 31 out of 31 recipient animals studied have shown immunologically specific partitioning of long-lived donor lymphoid cells immunized to SRBC or CRBC between lymph nodes challenged with these two antigens.

#### DISCUSSION

We have previously shown (1) that the loss of radioactive thymidine from a population of labeled lymphocytes follows a decay curve that is very similar

to the curve describing the disappearance of labeled cells radioautographically (2, 3), and therefore that long-lived lymphocytes can be studied radiochemically using a liquid scintillation spectrometer. We have also shown that the accumulation of radioactive thymidine in stimulated lymph nodes reflects the retention of long-lived cells and is not an artifact of thymidine reutilization. While most of this retention is nonspecific, a part of it is on the basis of immunological specificity (1). We have now extended these observations to a second pair of antigens that, while still thymus dependent (8, 9), provoke a response that is primarily immunoglobulin mediated rather than cell mediated.

We have shown first that there is a rapid accumulation of long-lived lymphocytes in lymph nodes reacting secondarily to CRBC. This accumulation is detectable within 8 hr of challenge and might be demonstrable even earlier in an adoptive transfer system where the background of nonrecirculating labeled cells would be lower. This response to CRBC seems to be more rapid than the response to *H-2*-compatible histocompatibility antigen (1). It is not clear whether this difference is related to CRBC reaching the draining node more rapidly, to differences in the strength of the antigens, or to differences between immunoglobulin-mediated and cell-mediated reactions. Others have also observed an accumulation of lymphoid cells in antigenically stimulated lymphoid organs (10-15) but not specifically the long-lived population. Evidence will be presented elsewhere that members of the rapidly dividing population of peripheral lymphoid cells show very little tendency to accumulate in antigenically stimulated lymph nodes (Emeson, Thursh, and Noble, manuscript in preparation). We have also shown that a chemically induced inflammatory reaction (turpentine) can cause local lymph nodes to retain long-lived cells but that the induction of phagocytosis within the lymph nodes with colloidal carbon does not. Again, others have shown that nonimmunological stimuli can cause cellular retention in lymph nodes (14), but not that this retention involves members of the long-lived population.

The most important result of the present studies is to confirm our observation that antigenically stimulated lymph nodes are capable of selectively accumulating long-lived lymphocytes of a particular immunological specificity. In our previous experiments involving allografts it was not possible to be certain that the observed immunologically specific accumulation of labeled thymidine in the regional lymph nodes was the direct result of the specific retention of recirculating lymphocytes in the lymph node. It remained possible that it reflected an accumulation of labeled cells (or breakdown products of cells) that reached the lymph nodes via the afferent lymphatics, and was thus secondary to events taking place in the allograft itself. In the present experiment, where there is no graft (or other lesion of cell-mediated hypersensitivity) present, it is much more likely that the immunologically specific accumulation of labeled thymidine reflects changes taking place within the lymph node itself. Furthermore, the present studies have shown that the immunologically

specific accumulation of long-lived cells in stimulated lymph nodes is not restricted to cell-mediated reactions, but probably occurs in all thymus-dependent immunological reactions. We are presently ascertaining whether a similar phenomenon also occurs in thymus-independent reactions.

In most of our work to date, we have been adoptively immunizing the recipient animals 24–72 hr before challenge, because we wished to allow time for the long-lived recirculating cells to come to equilibrium with the recipient's own lymphocytes and thus more closely approximate physiological conditions. The present studies have shown that the immunologically specific component can be demonstrated just as readily when the challenge is given 3 days before adoptive transfer. This indicates that the stimulus for specific retention is still present 3 days after primary challenge. It also indicates that, at least for these antigens, the only cells that are relevant in terms of immunologically specific localization are the long-lived cells that retain the ability to recirculate. Any other labeled cells that are present seem to lack immunological specificity and/or the ability to localize in stimulated lymph nodes.

While much remains to be learned about antigen-induced changes in lymphocyte recirculation, some tentative conclusions are now possible. It seems clear that after antigenic challenge, antigen (16) and/or sensitized lymphocytes (17) enter the regional lymph node via the afferent lymphatics. This initiates a sequence of events leading to a net increase in the number of recirculating cells present, in part by inhibiting their passage into the efferent lymphatics (18, 19). The present studies indicate that this antigen-induced sequence of events alters lymphocyte traffic in at least two different ways, one of which affects the passage of recirculating lymphocytes through the node irrespective of their specificity while the other affects only the passage of cells with specific reactivity to the antigen involved. The existence of these two separate mechanisms for the accumulation of recirculating cells in stimulated lymphoid organs has been previously postulated by others (15) and finds support in the experiments of Sprent, Miller, and Mitchell (20) and O'Toole and Davies (21). The functional significance of these mechanisms is obvious in the context of any selectional theory of acquired immunity that postulates that antigen-sensitive cells are limited in the number of specificities they can express. They provide a way in which a relatively small number of lymphoid cells of a particular specificity, distributed throughout the animal's recirculating pool, can be rapidly mobilized in order to initiate and amplify an immunological response to the local exposure to that antigen. Although direct proof is not yet available, there is good reason to believe (22, 23) that the specifically localizing lymphocytes observed here and elsewhere (1) are thymus-derived lymphocytes that: (a) function as specific antigen reactive cells (or memory cells), (b) proliferate locally (24), and (c) interact locally with antibody-forming cell precursors (bone marrow-derived lymphocytes) to facilitate the proliferation and differentiation of these cells.



## SUMMARY

The lymph nodes of mice actively or adoptively immunized to sheep RBC and/or chicken RBC selectively retain long-lived lymphocytes after challenge with the appropriate antigen. This retention is demonstrable within 8 hr of the time of stimulation, though it probably begins even before this, and it is essentially complete within the first 24 hr. A similar selective retention is seen in nodes regional to the injection of some nonimmunogenic substances such as turpentine, but not others such as colloidal carbon or syngeneic RBC.

In animals adoptively immunized to sheep and chicken RBC simultaneously, there is a preferential accumulation of the labeled long-lived lymphocytes of donors immunized to sheep RBC in lymph nodes challenged with sheep RBC, and a preferential accumulation of lymphocytes (labeled with a different radioisotope) from donors immunized to chicken RBC in lymph nodes challenged with this antigen. This immunologically specific component is demonstrable whether the antigen is given before or after adoptive immunization, suggesting that the only labeled cells capable of specific localization in this system are those cells that normally remain in the recirculating pool. In the present experiments, 31 out of 31 sets of antigenically stimulated lymph nodes have shown radiochemical evidence of immunological specificity in the distribution of donor lymphocytes between them, while corresponding sets of non-stimulated lymph nodes have shown only small random variations in the distribution of donor cells. Two different mechanisms are postulated whereby antigenic stimulation can alter the traffic of recirculating long-lived lymphocytes through stimulated lymph nodes. One affects recirculating cells of a particular immunological specificity, while the other affects recirculating cells without regard to their immunological specificity.

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