

THE SECONDARY IMMUNE RESPONSE TO A HAPTEN IN VITRO  
ANTIGEN CONCENTRATION AND THE CARRIER EFFECT\*

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The finding that the secondary immune response to a haptenic determinant is maximized by presenting the hapten on the carrier used for primary immunization has been termed the "carrier effect" (1-3). Recent investigations have indicated that secondary stimulation by the hapten on other carriers can be enhanced by immunization to the heterologous carrier or the transfer of cells from an animal immunized to the heterologous carrier (4-6). These findings have led to the postulate that in order to obtain haptenic stimulation two cells must interact, one specific for carrier determinants and one specific for the haptenic determinant.

This report describes analyses of secondary stimulation to a hapten in vitro. Secondary stimulation was carried out with 2,4-dinitrophenyllysine on hemocyanin (DNP-Hy),<sup>1</sup> the homologous carrier, and a variety of other carriers in a wide range of concentrations. The results indicate that stimulation was maximized by the use of Hy as the carrier; however, significant stimulation was observed with DNP on a variety of carriers including poly-L-lysine and poly-D-lysine, relatively nonimmunogenic carriers (7, 8). In addition, antigen concentrations presenting as little as  $10^{-13}$  M DNP were sufficient for stimulation using either homologous or heterologous carriers. A major difference between the presentation of the hapten on Hy as compared to other carriers, however, was observed at relatively high antigen concentrations. At antigen concentrations presenting  $10^{-6}$  M DNP, DNP-Hy was stimulatory, while on nonhomologous carriers this DNP concentration was inhibitory.

These results are discussed with a view to elucidating the mechanism of secondary stimulation and the role of cell-to-cell interactions in this response.

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<sup>1</sup> *Abbreviations used in this paper:* B $\gamma$ G, bovine  $\gamma$  globulin; B-lymphocytes, bone marrow-derived cells; DNP, 2,4-dinitrophenyllysine; DNP-lys-BAC, DNP-lysyl-bromacetyl cellulose; DNP-lys,  $\epsilon$ -DNP-L-lysine; HSA, human serum albumin; Hy, hemocyanin; PBS, sodium phosphate buffer; T-lymphocytes, thymus-dependent cells.

### Materials and Methods

*Antigens and Immunoabsorbants.*—Hemocyanin (Hy) was prepared by Sephadex G-200 chromatography (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) of the hemolymph of *Limulus polyphemus*. Human serum albumin (HSA) and bovine  $\gamma$ globulin (B $\gamma$ G) were obtained from Armour Industrial Chemicals Co., Chicago, Ill. Poly-D-lysine (75,000 mol wt) and poly-L-lysine (75,000 mol wt) were obtained from Miles-Yeda, Rehovot, Israel. Proteins were coupled to the 2,4-dinitrophenyl haptenic group by reacting 1 g of 2,4-dinitrobenzene sulfonate (Eastman Kodak Co., Rochester, N. Y.) with 3 g of protein (9). After extensive dialysis against 0.02 M sodium phosphate buffer (PBS), pH 7.2, and 0.15 M sodium chloride, these DNP-protein complexes were analyzed for nitrogen content by micro-Kjeldahl and optical density at 360  $\mu$  by a Zeiss PMQII spectrophotometer. Assuming a molar extinction coefficient for the DNP determinant of 17,400 (10), DNP-Hy was shown to contain 10 moles of DNP/100,000 g of Hy, DNP-HSA contained 22 moles of DNP/70,000 g of HSA, and DNP-B $\gamma$ G contained 21 moles of DNP/150,000 g of B $\gamma$ G. DNP-poly-D-lysine and DNP-poly-L-lysine were prepared according to the method of Kantor et al. (11). After extensive dialysis, these compounds were tested by nitrogen content and optical density. DNP-poly-D-lysine contained 22 moles of DNP/75,000 g of poly-D-lysine and DNP-poly-L-lysine contained 34 moles of DNP/75,000 g of poly-L-lysine.

$\alpha$ , $N$ [ $^3$ H] acetyl- $\epsilon$ -DNP-L-lysine was prepared as previously described as was the immunoabsorbant DNP-lysyl-bromacetyl cellulose (DNP-lys-BAC) (12).  $\epsilon$ -DNP-L-lysine (DNP-lys) was obtained from Yeda, Rehovot, Israel.

*Radioimmunoassay.*—The radioimmunoassay used to measure antibody concentrations and the purification of the mouse anti-DNP antibody used to standardize this assay have been previously described (13). 30  $\mu$ g of DNP-lys-BAC was used as the immunoabsorbent for each analysis and  $^{125}$ I-rabbit anti-mouse immunoglobulin Fab fragment (13) was used to detect bound anti-DNP antibody. 0.1 ml of culture fluid was used for each antibody analysis. The radioimmunoassay for anti-Hy antibody was also carried out as previously described (14).

*Mouse Immunizations.*—BALB/c mice 2–3 months of age were immunized by the intraperitoneal injection of 0.1 mg of DNP-Hy in complete Freund's adjuvant. Sera antibody concentrations, assayed by the radioimmunoassay, showed a maximum concentration of 0.3 mg/ml 2–4 wk after immunization. Sera anti-DNP antibody concentrations were less than 20 ng/ml 4–8 months after immunization when the animals were used as spleen cell donors or were secondarily immunized in vivo. Secondary immunizations were carried out by intraperitoneal injections with 50  $\mu$ g of DNP-Hy in PBS.

*Cell Transfers.*—Spleens were removed and cells were separated by a teflon pestle tissue homogenizer. Cells were washed and resuspended in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.). 30–40 million cells in 0.3 ml doses were injected into a tail vein of syngenic recipients irradiated with 900 R 24 hr previously. Non-immunized controls were obtained by injecting  $4 \times 10^7$  cells obtained from spleens of non-immunized mice.

*Fragment Cultures.*—24 hr after cell transfer spleens were removed and cut into 1–1.2 mm cubes. Culture conditions were similar to those previously described (14, 15). Each fragment was placed in 0.5 ml of Dulbecco's modified Eagle's medium supplemented with 50  $\mu$ l of horse serum from which gamma globulin was removed (Hyland Laboratories, Yonkers, N. Y.), 25  $\mu$ l of 10 day chick embryo extract (a gift from Dr. Jay Lash), 50 units of penicillin, and 50  $\mu$ g of streptomycin. Cultures were incubated in a moist chamber at 37°C in an atmosphere of 93% O<sub>2</sub> and 7% CO<sub>2</sub>. Media were removed and replaced at 2–3 day intervals for the duration of the cultures. Antigen was added to the culture fluid 24 hr after cultures were

initiated. Fragments were rinsed free of antigen containing culture fluid 2–4 days after antigen was added. For inhibition studies, the inhibitor was added to the fragment cultures at least 1 hr before antigen was added and was removed at the time of antigen removal.

Collected culture fluids were analyzed for anti-DNP and anti-Hy antibody content by the radioimmunoassay. Results are presented as the average amount of antibody produced per fragment per day. Included in this average were all fragments stimulated under the stated conditions in at least three separate experiments. In each experiment 4 fragments were utilized for each experimental point so that the data presented represented the results of at least 12 fragments for each point. In general a single experiment consisted of 100–120 fragments obtained from four to six recipient mice which had received pooled spleen cells from two donor mice.

*Equilibrium Dialysis.*—Equilibrium dialysis was carried out as previously described (12, 13). Sera and culture fluids were twice precipitated with 40% saturated ammonium sulfate and dialyzed against PBS before analysis.

#### RESULTS

Fig. 1 shows the amount of anti-DNP antibody released by fragments at various times after stimulation with 0.1 mg of DNP-Hy. Antibody was first detected at day 6–8 and reached a maximum at day 12–14. Such a response was observed in at least 80% of fragments tested. Antibody production continued for at least a month in these cultures. If no DNP-containing antigen was added to culture fluids or if cells from nonimmunized mice were used as donor cells, no anti-DNP antibody production was observed. Similar results were obtained when culture fluids from these fragments were analyzed for anti-Hy production. At least 80% of the fragments produced anti-Hy antibody with an average maximum release of 70 ng/day at day 14.

Stimulation by DNP-Hy was partially inhibited when carried out in the presence of relatively high concentrations of DNP-lysine or Hy. Table I summarizes the data obtained from cultures stimulated in the presence of free hapten or carrier. These results are consistent with those previously reported by several investigators (16–17).<sup>2</sup> Control studies indicated that  $10^{-5}$  M DNP-lysine did not reduce the amount of anti-Hy antibody produced by cultures stimulated with DNP-Hy.

Fig. 2 shows the effect on stimulation of variations in the carrier used in the stimulatory complex and the concentration of the various antigens used. Fig. 3 presents the same data calculated on the basis of DNP molarity. Maximum stimulation was obtained with 0.1  $\mu$ g/ml of DNP-Hy; however, significant stimulation was obtained with concentrations as high as 100  $\mu$ g/ml and as low as  $10^{-6}$   $\mu$ g/ml. Significant stimulation was also observed with DNP-HSA and DNP-B $\gamma$ G at concentrations as high as 1  $\mu$ g/ml and as low as  $10^{-6}$   $\mu$ g/ml. DNP-poly-L-lysine stimulated at concentrations as high as 0.03  $\mu$ g/ml and as low as

<sup>2</sup> Feldman, M. et al. 1970. Presented at the Third Sigrid Juselius Foundation Symposium. Helsinki, Finland.

$3 \times 10^{-7} \mu\text{g/ml}$  while DNP-poly-D-lysine was stimulatory at concentrations as high as  $2 \times 10^{-4} \mu\text{g/ml}$  and as low as  $2 \times 10^{-7} \mu\text{g/ml}$ . With all antigens DNP concentrations as low as  $10^{-13} \text{ M}$  were stimulatory. DNP on carriers other than

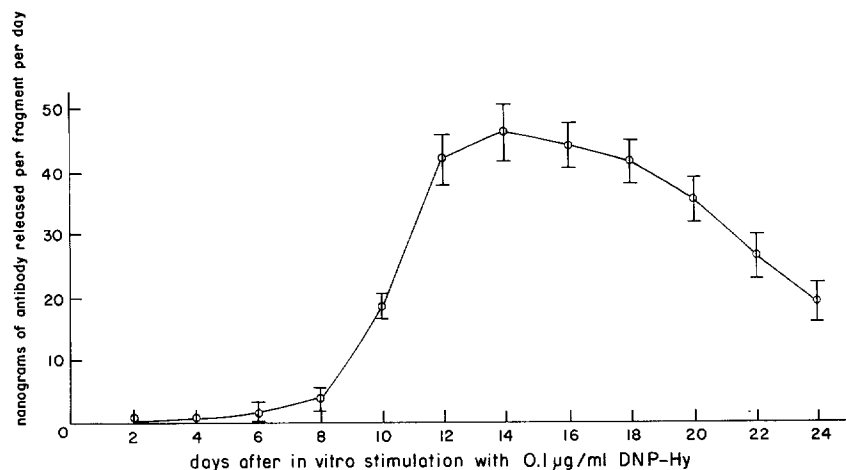


FIG. 1. The release of anti-DNP antibody by spleen fragment cultures with time after in vitro stimulation with  $0.1 \mu\text{g/ml}$  of DNP-Hy. Points represent arithmetic mean (with standard deviation) of data averaged from radioimmunoassays of culture fluids from fragment cultures from 10 separate experiments.

TABLE I  
*Inhibition of DNP-Hy Stimulation by DNP-Lysine and Hy*

Antigen	Inhibitor		Maximum amount of antibody released per culture per day (and SD)
	DNP-Hy	DNP-lys	
( $\mu\text{g/ml}$ )	( $\mu\text{g/ml}$ )	( $\mu\text{g/ml}$ )	( $\text{mg}$ )
0.1	—	—	$47 \pm 4$
1.0	—	—	$42 \pm 5$
10.0	—	—	$24 \pm 4$
0.1	$10^{-6}$	—	$26 \pm 6$
0.1	$10^{-5}$	—	$12 \pm 3$
1.0	$10^{-5}$	—	$18 \pm 4$
0.1	—	100	$14 \pm 4$
1.0	—	100	$8 \pm 3$
10.0	—	100	$4 \pm 2$

Hy at concentrations of  $10^{-6} \text{ M}$  and greater was not stimulatory. Table II shows the inhibitory effects of DNP on carriers other than Hy. It can be seen that the stimulatory effects of  $0.1 \mu\text{g/ml}$  of DNP-Hy could be inhibited by high concentrations of DNP on nonhomologous carriers. In all instances anti-Hy

antibody production by these fragments was essentially unaffected by the presence of such concentrations of these antigens.

Equilibrium dialysis studies showed that the binding of  $\alpha_1N$  [ $^3H$ ]-acetyl- $\epsilon$ -DNP-L-lysine at 7°C by antibody present in the serum of secondarily stimulated BALB/c mice, and that in the culture fluid of fragments stimulated with

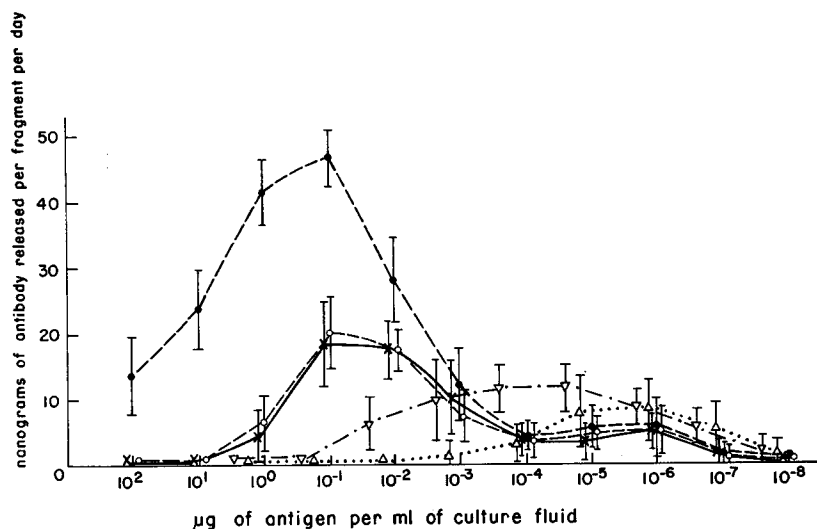


FIG. 2. The dependence of secondary in vitro stimulation on antigen concentration and hapten-carrier complex. Antigen concentration is presented as micrograms per milliliter. Each point represents the arithmetic mean (with standard deviation) of antibody released per fragment per day by at least 12 fragment cultures stimulated identically. Closed circles (●) represent stimulation with various concentration of DNP-Hy, open circles (○) represent stimulation with DNP-B $\gamma$ G, crosses (×) represent stimulation with DNP-HSA, triangles (△) represent stimulation with DNP-poly-D-lysine, and inverted triangles (▽) represent stimulation with DNP-poly-L-lysine.

1  $\mu\text{g}/\text{ml}$  or  $10^{-3}$   $\mu\text{g}/\text{ml}$  of DNP-Hy, were essentially the same. The  $K_a$  of the serum antibody was  $2.4 \times 10^7 \text{ M}^{-1}$  while that of antibody from pooled media of fragments stimulated with 1  $\mu\text{g}$  of DNP-Hy was  $2.1 \times 10^7 \text{ M}^{-1}$  and antibody produced by fragments stimulated with  $10^{-3}$   $\mu\text{g}/\text{ml}$  of DNP-Hy was  $2.6 \times 10^7 \text{ M}^{-1}$ .

#### DISCUSSION

This report describes an analysis of antigenic stimulation of the secondary immune response to DNP in vitro. The results indicate that: (a) maximum stimulation was obtained with DNP complexed to Hy, the carrier used for primary immunization, (b) stimulation was obtained with the hapten on non-

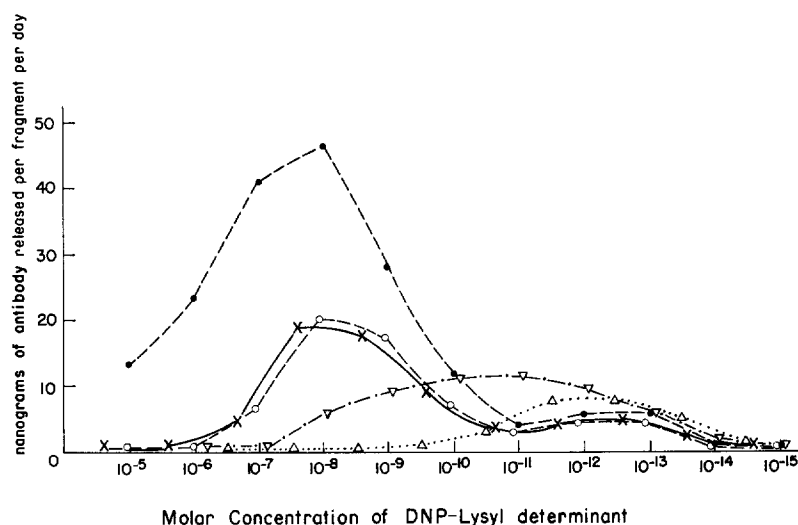


FIG. 3. The dependence of secondary in vitro stimulation on antigen concentration and hapten-carrier complex. Antigen concentration is presented as molar concentration of the DNP determinant. Each point represents the arithmetic mean of antibody released per fragment per day by at least 12 fragments. The data presented in this figure and the standard deviation of the calculated points are the same as in Fig. 2. As in Fig. 2, closed circles (●) represent stimulation with DNP-Hy, open circles (○) represent stimulation with DNP-B $\gamma$ G, crosses (×) represent stimulation with DNP-HSA, triangles ( $\Delta$ ) represent stimulation with DNP-poly-D-lysine, and inverted triangles ( $\nabla$ ) represent stimulation with DNP-poly-L-lysine.

TABLE II  
*The Inhibition of the In Vitro Response to DNP-Hy by DNP on Heterologous Carriers*

DNP-Hy	Antigen				Maximum amount of antibody released per culture per day (and sd)
	DNP-HSA	DNP-B $\gamma$ G	DNP-P-L-lys	DNP-P-D-lys	
		( $\mu\text{g}/\text{ml}$ )			(mg)
—	—	—	—	—	<0.5
0.1	—	—	—	—	47 $\pm$ 4
0.1	11	—	—	—	6 $\pm$ 2
0.1	110	—	—	—	<0.5
0.1	—	9	—	—	3 $\pm$ 2
0.1	—	90	—	—	<0.5
0.1	—	—	0.3	—	11 $\pm$ 5
0.1	—	—	3	—	<0.5
0.1	—	—	—	0.2	2 $\pm$ 1
0.1	—	—	—	2	<0.5

homologous carriers including DNP-poly-D-lysine, a poor immunogen, (c) stimulation was obtained with the hapten on homologous and nonhomologous carriers at antigen concentrations as low as  $10^{-6}$   $\mu\text{g}/\text{ml}$  ( $10^{-13}$  M DNP), and (d) at an antigen concentration of  $10$   $\mu\text{g}/\text{ml}$  ( $10^{-6}$  M DNP) and greater, DNP-Hy was stimulatory while DNP on nonhomologous carriers specifically inhibited the response to DNP.

Several investigators have reported the *in vivo* stimulation of anti-hapten antibody by hapten on nonhomologous carriers (18, 19). However, other investigators have found that hapten on the homologous carrier is a much better immunogen and have implied that specific recognition of carrier determinants enhances the affinity of hapten recognition (20).

The demonstration, in this report, that stimulation of a secondary response was obtained at equally low concentrations of the hapten on homologous and nonhomologous carriers indicates that the affinity of the hapten cell interaction was independent of the carrier molecule. The affinity of the antibody produced after stimulation with DNP-Hy in a concentration of  $10^{-3}$   $\mu\text{g}/\text{ml}$  ( $10^{-10}$  M DNP) was  $2.6 \times 10^7$   $\text{M}^{-1}$ . Since the hapten was presented in a multivalent form, it is possible that the affinity of cell-bound antibody for hapten was greatly enhanced by multivalent binding (21–24). Thus cell-bound antibody of an affinity of  $2.6 \times 10^7$   $\text{M}^{-1}$  for a monovalent hapten could bind a multivalent antigen with an affinity more than two logarithms higher. From the results presented here such multivalent interactions appear more likely to explain the stimulatory capacity of low antigen concentrations than would specific interactions with carrier determinants.

On the other hand, specific recognition of the homologous carrier appeared highly significant at higher antigen concentrations. DNP on nonhomologous carriers specifically inhibited the response to DNP at concentrations where DNP-Hy was stimulatory. Thus while a stimulatory interaction of the haptenic determinant in a polyvalent form and an immunocompetent cell may be possible, the general mechanism of antigenic stimulation is probably more complex.

Mitchison and his co-workers consider the carrier effect as a function of thymus-dependent cells (T-lymphocytes) which recognize carrier determinants. Such cells presumably bind the hapten-carrier complex and present the hapten in a concentrated form to bone marrow-derived cells (B-lymphocytes) which recognize the hapten and upon stimulation produce antibody against it (3). Two cells which recognize determinants on the same antigen molecule could possibly form a highly stable complex if both shared in the simultaneous binding of numerous antigen molecules (21–24). Cell-to-cell interactions could thus play a role in antigenic stimulation if such stable complexes between antigen and cells were necessary. Such interactions would be minimized at very low antigen concentrations where too few antigen molecules would be available for stable cross-linking and at very high antigen concentrations where cross-linking would

be minimized by blockage of antigen binding sites. Stimulation of anti-DNP antibody production, *in vitro*, by low concentrations of hapten on homologous and nonhomologous carriers could result from interactions of two cells recognizing DNP. Since this determinant is polyvalent it may be capable of simultaneous interaction with cell-bound antibody of more than one cell. Because of its polyvalence, its interaction with cell-bound antibody may be of relatively high affinity. Thus saturation of DNP receptor sites may be reached at relatively low antigen concentrations. Receptor sites recognizing carrier determinants, however, may still be available for cross-linkage at higher antigen concentrations. Thus the recognition of Hy would enhance stimulation of anti-DNP cells at high antigen concentrations. This is consistent with the finding that high concentrations of Hy-inhibited stimulation with DNP-Hy.

This mechanism for stimulation through cell-to-cell interaction does not differentiate between the types of cells involved. It is possible that the interaction of two B-lymphocytes could be mutually stimulatory. Such a mechanism has been postulated for the stimulation of "mono-focal" antibody production where stimulation shows enhancement by carrier recognition but can occur in the absence of carrier specific cells (14). On the other hand, the presence of T-lymphocytes recognizing determinants on the antigen molecule could serve to increase the probability of productive interactions of antigen and hapten specific B-lymphocytes. This would be consistent with the marked enhancement of antigenic stimulation generally observed in the presence of carrier recognition. An interesting correlate of the above hypothesis is the possibility that tolerance may be produced by simple antigen-cell interactions or by the formation of unstable complexes between two cells cross-linked by only a few antigen molecules. Such a state would be obtained at very high or very low antigen concentrations as has been described for the induction of the tolerant state (25).

#### SUMMARY

The *in vitro* secondary stimulation of the production of anti-hapten antibody has been analyzed with a view to elucidating the role of the carrier molecule and cell-to-cell interactions in this response. Stimulation was carried out on fragment cultures of the spleens of irradiated BALB/c mice which had been reconstituted with  $3-4 \times 10^7$  spleen cells from isologous mice previously immunized with DNP-Hy. The results indicated that the response was maximized by stimulation with DNP-Hy, the homologous complex, however anti-DNP antibody production could be obtained by stimulation with DNP on several nonhomologous carriers including poly-D-lysine, a poor immunogen. The results also indicated that while DNP-Hy and DNP-nonhomologous-carrier complexes were stimulatory at equally low DNP concentrations, at DNP concentrations over  $10^{-6}$  M DNP-Hy was stimulatory, while DNP on non-



homologous carriers was inhibitory. The results are interpreted as indicating that: (a) the affinity of the antigen-cell interaction is more likely determined by multivalent binding than by carrier recognition, (b) that a stimulatory interaction of a polyvalent antigen with a B-lymphocyte cannot be excluded, (c) that if cell-to-cell interaction is necessary for stimulation, then both cells may recognize the same determinant, and (d) that the marked enhancement of antigenic stimulation attributable to carrier recognition may result from stimulatory interactions of cells recognizing different antigenic determinants. A mechanism is postulated whereby stimulation is dependent on the formation of stable complexes resulting from two cells sharing in the binding of numerous antigen molecules. Cells recognizing carrier determinants would increase the probability of such interactions particularly at high antigen concentrations.

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