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Immune responses in mice infected with lactic dehydrogenase virus

I. ANTIBODY RESPONSE TO DNP-BGG AND HYPERGLOBULINAEMIA IN BALB/c MICE

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Summary. The humoral immune response to DNP-BGG of BALB/c mice acutely infected with lactic dehydrogenase virus (LDV) has been investigated. Virus-infected mice injected with antigen in saline exhibit a greater anti-DNP response than uninfected controls. When this antigen is presented in Freund's complete adjuvant (FCA) the anti-DNP response is greater than obtained with antigen in saline, but significant differences between infected and uninfected controls are not observed. These data are consistent with the view that acute LDV infection can have an adjuvant-like effect when this T-dependent antigen is introduced in saline.

In addition, the effect of viral infection on plasma Ig class and subclass levels has been investigated. LDV infection leads to a gradual increase in plasma Ig concentration. This effect is restricted to the IgG2a subclass in most animals, but occasionally is restricted to IgG1. The mechanisms responsible for these changes have not been delineated.

INTRODUCTION

Immune responses involve the interaction of three cell types: B cells, T cells, and macrophages. Murine lactic dehydrogenase virus (LDV) infection serves as an experimental model for study of these interactions, as the host cell for this virus appears to be specifically the mouse macrophage (Rowson & Mahy, 1975), and acute LDV infection has been reported to decrease cellular immunity (Howard, Notkins & Mergenhagen, 1969) and increase humoral responses (Notkins, Mergenhagen, Rizzo, Scheele & Waldmann, 1966). LDV is a small (35 nm) RNA (single-stranded 48S) enveloped virus composed of three structural proteins (Michaelides & Schlesinger, 1973). The host range of the virus is limited to the mouse; the infection is permanent and the animals exhibit no obvious signs of illness. Notkins *et al.* (1966) have presented evidence that mice immunized with human γ-globulin in saline one day after LDV infection were able to produce more antibody (as shown by immune elimination of [^{131}I]HGG injected some time later) than uninfected controls. They also demonstrated an elevation of serum immunoglobulin levels in LDV-infected germ-free mice. The present work further explores the observation that acute LDV infection leads to an increased humoral response. The anti-DNP antibody response of LDV-infected mice was compared to that of uninfected controls.

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Also, plasma immunoglobulin levels were monitored in these animals to determine if LDV infection altered their isotype (class and subclass) distribution.

MATERIALS AND METHODS

Animals

BALB/c AnN female mice 8–9 weeks of age were used throughout these experiments. Periodic testing of our animals has always shown them to be free of endogenous LDV. Blood was collected from the tail into heparinized tubes, and plasma harvested following centrifugation.

LDV

This virus was originally obtained from the sera of BALB/c mice bearing plasmacytoma MOPC 315 (Michaelides & Schlesinger, 1974), in which LDV apparently was a passenger. Pools of infectious virus were prepared from plasma of BALB/c mice infected 24 h previously with 2×10^6 ID_{50}/mouse i.p. This same virus dose and route was used to experimentally infect mice for these studies, unless otherwise noted. LDV was assayed according to Riley (1968).

DNP-BGG

DNP_{48}-BGG was prepared according to Eisen, Belman & Carsten (1953). Mice received a 250 μg dose of this antigen in 0·1 ml, given i.p. either in saline or emulsified with Freund's complete adjuvant. For secondary immunization, (2°) the same antigen dose was given in saline 42 days after primary immunization (1°).

Class and subclass-specific antisera

Goat anti-mouse immunoglobulins specific for IgG1, IgG2a, IgG2b, IgA, or IgM were purchased from Gateway Immunosera Co., Cahokia, Illinois. They were tested for monospecificity by double diffusion and immunoelectrophoresis against all other mouse Ig classes and subclasses and for the presence of any antibodies to other murine serum proteins.

Class and subclass-specific mouse Ig's

Ig standards for radial immunodiffusion (RID) included purified paraproteins MOPC 21 (γ1, κ); LPC-1, UPC-10, RPC-5 (γ2a, κ); MOPC 11 (γ2b, κ); MOPC 104E (μ, λ_1); and MOPC 315 (α, λ_2). Their purity was tested by double diffusion against monospecific class and subclass specific antisera; in addition they were tested against antisera to whole mouse serum. Protein 315 was purified from the serum of BALB/c An mice carrying this plasmacytoma according to Underdown, Simms & Eisen (1971). Proteins UPC-10 and RPC-5 were purchased from Bionetics, Kensington, Maryland. The other Ig standards were purchased from Gateway Immunosera Co., Cahokia, Illinois.

Antigen-binding capacity (ABC) of mouse plasma for ε-DNP-L-lysine

The Farr technique (Farr, 1958) as modified by Celada (1966) was used. [^{3}H]ε-DNP-L-lysine was obtained from New England Nuclear with a sp. act. of 1·1 Ci/mmole. A fixed amount of [^{3}H]ε-DNP-L-lysine (50 μl of 4×10^{-8} M) was mixed with an equal volume of test plasma. This plasma was used either undiluted, or diluted in PBS pH 7·1 containing 20% normal mouse plasma. Three dilutions of each test plasma were employed: 1/10, 1/40 and 1/60; or undiluted, 1/10 and 1/100. The hapten-antibody mixture was incubated for 1 h at 4°; then 200 μl of 66% $(NH_4)_2SO_4$ was added to every tube, mixed and incubated for 2 h at 4°. The tubes were centrifuged at 1400 *g* for 20 min at 4°; two 100-μl samples of the supernatant were withdrawn, each added to 10 ml scintillation fluid and radioactivity determined in a liquid scintillation spectrometer. Tubes were set up in duplicate and controls for nonspecific binding were included in each experiment. ABC was expressed as the fraction of or multiple of 20 picomol (2×10^{-11} mol) [^{3}H]ε-DNP-L-lysine bound by 1 ml of undiluted plasma.

Radial immunodiffusion (RID)

Plasma Ig levels of were determined according to the RID method of Mancini, Carbonara & Heremans (1965). Isotype-specific antiserum was incorporated into 1% agarose in 0·05 M barbital buffer, pH 8·6. Lantern slides (10·1 × 8·3 cm) were layered with 15 ml agarose each, and thirty wells (3 mm each) made per slide. Subclass or class standards as well as pooled normal mouse plasma were included on each slide. Slides were incubated in a humidified container at room temperature for 4 days (IgG subclass determinations) or for 7 days (IgM and IgA determinations). They were then washed, dried, and stained with amido black. For

quantification, the slides were magnified 2·2-fold, the outline of each precipitin circle marked on graph paper, and these areas cut out and weighed. Each sample was set up in duplicate and the standard deviation was 4·3%. This was calculated by taking one set of duplicate immunodiffusion plates at random and expressing the differences of duplicates from their mean as percent differences. Each of these per cent differences was equated to $(x-\bar{x})$ and the standard deviation was calculated from the following equation:

$$\text{s.d.} = \sqrt{\frac{(x-\bar{x})^2}{n-1}}.$$

A paraprotein other than the one used to generate the isotype-specific antiserum was included as the class or subclass standard for IgA and IgG2a determinations, but no standards other than the immunogen were available to us for IgM, IgG2b and IgG1 measurements. Therefore, our reported levels of IgM, IgG2b, and IgG1 may be overestimates of the actual plasma concentration of these immunoglobulins. For example, IgG2a is overestimated 2·6-fold when homologous antigen is used as the standard, probably due to the presence of both anti-isotype and anti-idiotype antibody. However, in spite of uncertainty as to the absolute amounts of these latter isotypes, relative changes in Ig concentration for uninfected *vs* infected mice are expressed by this method.

Catabolism of [^{125}I]*IgG2a*

Purified protein LPC-1 (γ2a, κ) was iodinated with solid state lactoperoxidase according to the procedure of David & Reisfeld (1974). ^{125}I carrier-free was purchased from Schwarz/Mann, Orangeburg, New Jersey (sp. act. 15–17·4 Ci/mg). Lactoperoxidase was obtained from Calbiochem, La Jolla, California. Sepharose 4B-200 was purchased from Sigma Chemical Co., St Louis, Missouri. The iodinated LPC-1 had a sp. act. of 30 μCi/mg. Two groups of 9-week-old mice (six mice per group) received 50 μg of [^{125}I]LPC-1 per mouse i.p. on day 0; one group had been infected with LDV on the previous day. All were given drinking water with 0·01% KI and 0·45% NaCl prior to and throughout the experiment, to reduce the uptake of ^{125}I by the thyroid gland. Twenty-four hours after injection of [^{125}I]LPC-1, all mice were bled (about 0·1 ml blood) and duplicate 10 μl plasma-samples from each mouse counted in a gamma spectrometer. Radioactivity in each plasma sample was taken to correspond to 100% for that mouse. Each group of six mice was divided into three subgroups for further bleedings, so that each subgroup was bled once every 3 days during the course of this 10-day experiment. In order to compensate for possible changes in counting efficiency and to correct for isotope decay, standards prepared at the outset of the experiment were counted each day along with that day's experimental plasma samples.

RESULTS

(a) Effect of acute infection with LDV on antibody production

The experimental protocol is shown in Table 1. Four groups of mice were used, five mice to a group. On day 0, groups I and II were injected with DNP-BGG in saline while groups III and IV received the same amount of antigen in Freund's complete adjuvant (FCA). Groups II and IV had been infected with LDV one day prior to this antigenic stimulation. All mice were bled on days 7 and 21. They received a second injection of DNP-BGG in saline on day 42, and were bled again on days 46 and 52, i.e., days 4 and 10 after this second immunization.

The ABC of individual mouse plasma was deter-

Table 1. Effect of LDV-infection on anti-DNP antibody production: experimental protocol

Group	LDV	DNP-BGG-1°		Animals bled		DNP-BGG-2°	Animals bled	
		saline	FCA			saline		
	Day −1	Day 0		Day 7	Day 21	Day 42	Day 46	Day 52
I	−	+	−	+	+	+	+	+
II	+	+	−	+	+	+	+	+
III	−	−	+	+	+	+	+	+
IV	+	−	+	+	+	+	+	+

Table 2. ABC of plasma from individual mice

Group	LDV†	days after 1° immunization		days after 2° immunization*	
		7	21	4	10
1° DNP-BGG in saline					
I	−	0·125±0·008‡	0·248±0·070	<0·236±0·024	3·284±0·976
II	+	0·884±0·182	0·644±0·075	0·776±0·129	12·996±6·837
		P§<0·005	P<0·005	P<0·005	n.s.¶
1° DNP-BGG in CFA					
III	−	7·25±2·17	59·80±12·20	65·28±11·29	276·28±50·08
IV	+	5·62±1·90	32·88±9·09	72·80±14·99	216·40±54·01
		n.s.	n.s.	n.s.	n.s.

* 2° Immunization on day 42.
† LDV infection 24 h prior to 1° immunization.
‡ Arithmetic mean±s.e.m. from five mice.
§ To calculate P, the data from group I were compared with group II; the data from group III were compared to group IV.
¶ Not significant differences; in all cases $P>0·10$.

mined, and Table 2 summarizes these findings. When the two groups which received the antigen in saline were compared, the antibody response of the LDV-infected mice (group II) was significantly greater than that of the uninfected controls (group I) during the primary response ($P<0·005$ for 1° day 7 and 21); this was also true for the early part of the secondary response but not later ($P<0·005$ for 2° day 4 but $P>0·10$ by day 10 of 2°). As expected, mice which received the antigen in FCA (groups III and IV) gave a much better antibody response than mice which had received the antigen in saline (groups I and II). A comparison of the antibody response to antigen in FCA between uninfected controls (group III) and LDV-infected mice (group IV) revealed no significant differences ($P>0·10$) in either the primary or secondary response.

(b) Effect of infection with LDV on Ig levels

The levels of immunoglobulins in the plasma of individual mice from the DNP-BGG experiment during the secondary response were measured by radial immunodiffusion. Table 3 summarizes these results, and includes for comparison Ig levels from normal mouse plasma pools. The most consistent observation is the increased level of IgG2a in the LDV-infected animals (groups II and IV) when compared to that in the uninfected controls (groups I and III). This increase appears to be independent of the level of anti-DNP antibody present. The other significant difference is the greater level of IgG1 in group III when compared to group IV on 2° day 4 ($P<0·005$); however this difference is smaller 6 days later ($P<0·05$ on 2° day 10).

To determine if the increased IgG2a level observed in LDV-infected, DNP-immune mice is also seen in non-immune infected mice, chronically-infected animals were bled at the age of 12–14 months and their immunoglobulin profiles compared to uninfected controls. The data from this experiment are summarized in Table 4. As a group these LDV-infected mice demonstrated a significantly greater ($P<0·005$) level of IgG2a than normal controls. Within this group, however were two mice with a low IgG2a profile (2–3 mg/ml) which exhibited principally an increase in IgG1 (5·5 mg/ml). Two other animals in this group had a lower IgG2a level (2–3·5 mg/ml) than the average for this group as a whole, without a concomitant increase in the level of any other Ig class or subclass. The remaining six mice from this group, i.e. 60% of these LDV-infected animals, exhibited IgG2a levels higher than observed on either day 4 or 10 after 2° immunization of infected DNP-immune mice (Table 3). In these six mice the level of IgG1 was not elevated (0·7–2·2 mg/ml). To determine how soon after infection with LDV the increase in IgG2a occurs, a group of mice were bled 4 days after virus infection and their level of IgG2a compared with controls of the same age. In thirteen uninfected mice the mean level of IgG2a (mg/ml±s.e.m.) was 1·50±0·088; in seven

Table 3. Ig levels (mg/ml) in plasma mice during the secondary response to DNP-BGG

Group	IgG2a		IgG2b		IgG1		IgM		IgA	
	Day*		Day		Day		Day		Day	
	4	10	4	10	4	10	4	10	4	10
I	1·85±0·16†	1·66±0·16	0·16±0·01	0·18±0·03	2·07±0·19	1·28±0·10	0·95±0·11	1·22±0·07	0·20±0·03	0·15±0·01
II	3·00±0·22	2·86±0·23	0·17±0·02	0·18±0·02	1·82±0·21	1·10±0·07	1·02±0·06	0·96±0·11	0·21±0·03	0·13±0·01
	P‡<0·005	P<0·005	n.s.§	n.s.	n.s.	n.s.	n.s.	P<0·05	n.s.	n.s.
III	2·10±0·23	2·18±0·20	0·29±0·03	0·29±0·03	2·98±0·19	2·34±0·18	1·08±0·04	1·34±0·11	0·28±0·03	0·19±0·02
IV	3·10±0·11	3·24±0·14	0·24±0·03	0·26±0·03	1·86±0·09	1·78±0·18	0·96±0·07	1·08±0·10	0·20±0·01	0·15±0·01
	P<0·005	P<0·005	n.s.	n.s.	P<0·005	P<0·05	P<0·1	P<0·1	P<0·05	P<0·05
Normal¶	1·55		0·15		1·01		1·20		0·16	

* Day after 2° immunization; equivalent to days 46 and 52 after 1° immunization.

† Arithmetic means±s.e.m. from five mice.

‡ To calculate P, the data from group I were compared to that of group II; the data from group III were compared to that of group IV.

§ Not significant differences; in all cases P>0·10.

¶ Averages from results with pools of normal mouse plasma.

Table 4. Ig levels (mg/ml) in plasma of mice 12–14 months of age

LDV*	No. mice	IgG2a	IgG2b	IgG1	IgM	IgA
+	10	4·90±0·65† (2·19–7·28)	0·31±0·04 (0·14–0·52)	2·23±0·56 (0·7–5·6)	0·65±0·04 (0·48–0·89)	0·24±0·01 (0·22–0·27)
−	5	1·88±0·32 (1·30–2·72)	0·26±0·02 (0·21–0·32)	1·38±0·10 (1·0–1·6)	0·63±0·05 (0·48–0·82)	0·33±0·06 (0·23–0·54)
		P‡ < 0·005	n.s.§	n.s.	n.s.	P < 0·05

* Mice were infected with LDV at 2 months of age.

† Arithmetic mean±s.e.m.; numbers in parentheses denote range.

‡ To calculate P, the data from the LDV-infected group was compared with the data from the uninfected group.

§ No significant differences; in these cases $P > 0·10$.

LDV-infected animals the level of IgG2a was 1·32 ± 0·104. Therefore, no elevation in plasma IgG2a is evident this early (4 days) after infection. The sum of these observations, while not proof, supports the view that LDV infection results in a gradual and persistent elevation of IgG2a in most mice.

(c) Catabolism of [^{125}I]IgG2a in normal and LDV-infected mice

Since it is known that the catabolism of lactic dehydrogenase enzyme is decreased in LDV-infected animals (Notkins & Scheele, 1964), the possibility arose that the increased level of IgG2a observed here in the chronically-infected mice was due to a decreased rate of its catabolism.

To evaluate this possibility a purified myeloma protein (LPC-1; γ2a, κ) was iodinated, injected i.p. into six normal mice and six LDV-infected mice, and its catabolism followed for 10 days *in vivo*. The results of this experiment are summarized in Fig. 1. A linear regression line was calculated for each subgroup and half-lives (T 1/2) calculated from the slope. No significant difference was found ($P > 0·4$) when the T 1/2's obtained for each of the three subgroups of uninfected mice were compared to those of the LDV-infected mice. The T 1/2's obtained from linear regression lines, calculated when the data from each group of six mice was pooled, was 4·96 for the uninfected controls, and 5·28 for the group of LDV-infected mice. The T 1/2 for IgG2a reported previously is 5 days (Spiegelberg, 1974).

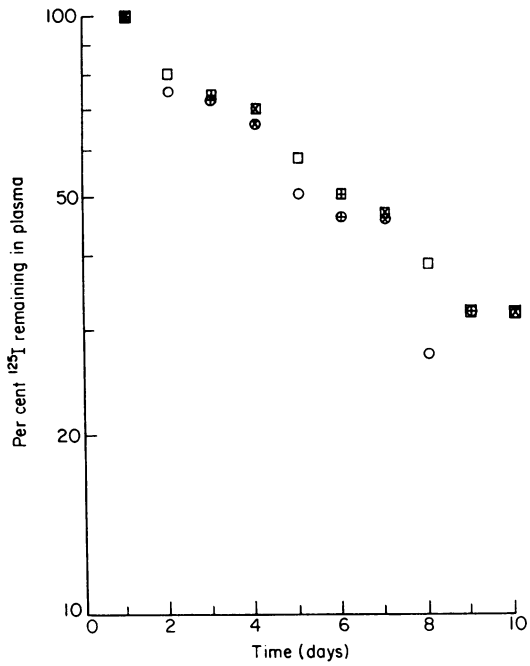

Figure 1. Rate of catabolism of [^{125}I]IgG2a in mice. The days on the abscissa refer to the time in days after the i.p. injection of [^{125}I]IgG2a. Uninfected animals (six per group) are represented as circles and LDV-infected animals (six per group) as squares. The open symbols refer to the subgroup bled on days 1, 2, 5 and 8. The symbols enclosing a + to the subgroup bled on days 1, 3, 6, 9 and those enclosing an × to the subgroup bled on days 1, 4, 7 and 10. Since all mice were bled on day 1 and those values represent 100% of their radioactivity, these symbols are coincident.

DISCUSSION

These experiments were undertaken to extend the findings of Notkins *et al.* (1966) that infection with LDV leads to hyperglobulinaemia and that acute infection has an adjuvant-like effect. To test for

the adjuvant-like effect, normal as well as acutely LDV-infected mice were injected with DNP-BGG either in saline or in FCA. When DNP-BGG was given in saline, the plasma ABC of mice infected with LDV was much greater than that of uninfected controls ($P < 0{\cdot}005$ for days 7 and 21 after primary immunization). When the antigen was given in FCA, the plasma ABC was considerably greater and no significant differences in ABC were evident between the normal and the LDV-infected animals. Therefore, infection with LDV can indeed have an adjuvant-like effect, as was noted when this T-dependent antigen was injected in saline. However, in the presence of FCA, which itself maximizes this antibody response, no further increase in ABC was obtained by LDV infection.

All groups responded to a secondary challenge with antigen in saline to give an anamnestic response. The highest anti-DNP responses were encountered in the two groups that had been primed with the antigen in FCA, but no significant differences were observed between these two groups, i.e. between the LDV-infected mice and their uninfected controls. The secondary responses of the two groups which had been primed with antigen in saline indicate that during the early part of the secondary response, i.e. day 4, the LDV-infected mice responded better than their uninfected controls ($P < 0{\cdot}005$). However, by day 10 the variation in response within each group was large enough to make this difference between the two groups less significant ($P = 0{\cdot}20$). Therefore the adjuvant-like effect of LDV infection observed during the primary immune response may not be maintained as the secondary response progresses. Enhancement of humoral immune responses has not been observed when immunization occurs during the chronic phase of LDV infection (Rowson & Mahy, 1975). Since our secondary immunization occurred during chronic viral infection, it is probable that any enhancement we observed in the secondary response is a consequence only of timing the primary immunization during the acute phase of viral infection.

To examine more extensively the report of hyperglobulinaemia after LDV infection, the level of each immunoglobulin class or subclass in the plasma of virus-infected animals was compared to that of uninfected controls. When the animals previously immunized with DNP-BGG were examined (Table 3), the most significant difference that correlates with LDV infection is the increase in IgG2a. This is evidenced by the higher level of this Ig subclass in groups II and IV as compared to either group I or III. These mice had been infected with virus about 50 days earlier and any interpretation of the results was complicated by the additional effects of immunization. In order to substantiate that viral infection alone results in an increase in serum IgG2a and to provide information relating to the temporal aspects of this change, one group of mice was tested 4 days after LDV infection and another group was tested 10–12 months later. There was no increase in IgG2a in mice 4 days after infection with LDV; so this increase occurs sometime between days 4 and 46 and is probably gradual. Mice chronically infected with LDV, i.e. those tested 10–12 months later, generally exhibited higher levels of IgG2a (Table 4) than either their uninfected controls or most of the DNP-immune mice. This supports the conclusion that IgG2a apparently continues to increase with time after virus infection.

The increased IgG after LDV infection could be due to an increased rate of its synthesis or to a decreased rate of catabolism. Since it is well documented (Notkins & Scheele, 1964) that LDV infection leads to a decreased rate of catabolism of many enzymes, the latter possibility was explored. The clearance of [^{125}I]IgG2a was measured in mice acutely infected with LDV and was compared to that in uninfected controls; our focus was restricted to this subclass since its increase was the most frequently observed. When the data presented in Fig. 1 was analysed statistically, no significant difference can be seen in the rate of catabolism of [^{125}I]IgG2a between the normal and LDV-infected mice. The clearance rates were T 1/2 = 4·96 days for normal mice and T 1/2 = 5·28 days for the LDV-infected animals ($P > 0{\cdot}4$). In the literature the T 1/2 reported for IgG2a is 5 days (Spiegelberg, 1974). It is difficult to evaluate whether such a small difference in catabolic rates, even though statistically insignificant, might result in the change in IgG2a levels observed by day 50. The rate of change in plasma appears to be slow, and minor perturbations in other parameters such as synthetic rates, distribution volumes, etc., might contribute to changes in steady-state levels of Ig's. The criticism might be justifiably made that the interval after infection (2–12 days) at which catabolic rates were measured is inappropriate and chronically-infected animals should have been used. However, it is well known (Fahey & Robinson, 1963) that an increase in the

level of any Ig will increase its rate of catabolism; results from such an experiment therefore might be even more difficult if not impossible to interpret.

In addition to the previously considered possibility that IgG2a elevation in LDV-infected mice is due to a decreased catabolic rate, at least two other alternatives can be considered. One is that the virus itself or some viral component leads to a polyclonal, i.e. not antigen specific, activation of immunoglobulin synthesis and secretion, usually restricted to IgG2a. There are precedents which are compatible with this hypothesis, notably the non-specific increase in IgE which occurs after helminth infections and does not reflect the IgE antibody response to parasite antigens (Ishizaka, Urban & Ishizaka, 1976). A second possibility is that the hyperglobulinaemia represents anti-LDV antibody that is usually clonally restricted in BALB/c mice to the IgG2a subclass. If the restriction to IgG2a is not complete and in some mice the anti-LDV happens to be IgG1, this would explain the large increase in IgG1 in two out of ten mice chronically infected with LDV. This clonal restriction of anti-LDV to IgG2a would be analogous to the response of BALB/c mice to α-1,3 linked dextran (Cohn & Weigert, 1975) which is limited to antibodies that contain λ chains. Experiments to differentiate between these two explanations for the observed increased IgG2a in LDV-infected mice are currently being conducted.

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