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Immunology

Formation of IgE-binding factors by human T-cell hybridomas

(Fc receptors/T-cell factor/IgE response)

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ABSTRACT **Normal human T cells that proliferated in the presence of interleukin 2 (IL-2) formed IgE-binding factors when incubated with human IgE. These cells were then fused with a mutant of the human T-cell line CEM. Incubation of five hybridomas with human IgE or culture of the cells in IgE-coated wells resulted in the formation of IgE-binding factors. One hour of incubation with 10 μg of human IgE per ml was sufficient to induce the hybridomas to form IgE-binding factors. Polymerized IgE was much more efficient than monomeric IgE for the induction of the factor formation. As little as 10 ng of IgE dimer per ml was sufficient to induce factor formation. The IgE-binding factors produced by the hybridomas bound to human IgE-coated Sepharose and were recovered from the beads by elution at acid pH. The factors had low affinity for rat IgE but failed to bind to human IgG. The IgE-binding factors formed by four hybridomas had a M_r between 25,000 and 30,000, whereas one hybridoma formed IgE-binding factors of M_r 30,000 and M_r 15,000. The IgE-binding factors formed by all of the hybridomas had affinity for concanavalin A, indicating that the factors are glycoproteins.**

Previous studies from our laboratories have demonstrated the existence of rat T-cell factors that have affinity for homologous IgE and selectively regulate the IgE response. One of the IgE-binding factors potentiated the IgE response of rat lymphocytes (1), whereas another IgE-binding factor suppressed the IgE response (2). Both factors have a M_r of ≈15,000 but they appear to differ in their carbohydrate moieties. The IgE-potentiating factor has affinity for lentil lectin and concanavalin A (Con A). The IgE-suppressive factor failed to bind the lectins but has affinity for peanut agglutinin (PNA) (3). Subsequent experiments revealed that human T lymphocytes could produce IgE-binding factors, which were detected by the ability to inhibit rosette formation of $Fc_\epsilon R^+$ lymphocytes with IgE-coated ox erythrocytes (4). Activated lymphocytes obtained by mixed lymphocyte culture, as well as normal human T cells that proliferated in the presence of interleukin 2 (IL-2), formed IgE-binding factors upon incubation with IgE. However, the yield of the factors in these systems was too small for their characterization. Because several investigators have succeeded in obtaining human T-cell hybridomas that produce a variety of lymphokines (5–7), the present experiments were undertaken to construct human hybridomas that can produce IgE-binding factors. Normal human T cells that proliferated in IL-2-containing medium were incubated with human IgE, and the cells were fused to a subline of CEM. The results show that some of the hybridomas produce IgE-binding factors upon incubation with IgE.

MATERIALS AND METHODS

Immunoglobulins. Human IgE was isolated from the serum of a patient with IgE myeloma, PS, by procedures previously described (8). Monoclonal rat IgE was purified by the method of Isersky *et al.* (9) from the ascitic fluid of Lou/c rats infected with IR 162 immunocytoma (10). Human IgG was purified from normal human serum by precipitation with ammonium sulfate, followed by chromatography on DEAE-cellulose. Human IgE was polymerized by using a crosslinking reagent, dimethylsuberimidate (Sigma), as described by Finbloom and Metzger (11). The protein was applied to an ACA 22 column (2.5 × 90 cm) tandem to an ACA 34 column to obtain polymer, dimer, and monomer fractions. A portion of IgE-dimer was labeled with ^{125}I by using chloramine-T. Monomeric human IgE, rat IgE, or human IgG was coupled to Sepharose CL-4B (Pharmacia) by the method described by Conrad and Froese (12); 8–10 mg of Ig was coupled to 1 ml of activated Sepharose.

Formation of T–T Hybridomas. Normal T cells that proliferated IL-2-containing medium (described below) were fused with a hypoxanthine guanine phosphoribosyltransferase-deficient mutant of human lymphoblastoid cell line CEM. The subline of CEM (BUC) was kindly supplied by J. D. Stobo (University of California, San Francisco) and is OKT 4^+ or OKT 8^- HLA-DR^- (J. D. Stobo, personal communication). Equal numbers of human T cells and BUC cells were pelleted together and fused by using polyethylene glycol M_r 4000 (Sigma). Detailed procedures of cell fusion have been described (13). After fusion, cells were resuspended in hypoxanthine/aminopterin/thymidine (HAT)-containing medium, and 5×10^4 cells were seeded in each well of 96-well plates. Culture medium was complete Dulbecco's modified Eagle's medium (DME medium) (13). Cells were kept in HAT/DME medium for 3 weeks until clones appeared and the cells were maintained in complete DME medium with biweekly subculture. The subcloning of hybridomas was carried out in soft agar.

Cell Cultures. Culture medium was RPMI 1640 medium supplemented with 10% fetal calf serum, 3 mM L-glutamine, 50 μM mercaptoethanol, and antibiotics. The mononuclear cells were obtained from normal human peripheral blood by centrifugation on Ficoll-Paque and cultured in RPMI 1640 culture medium supplemented with human IL-2. Growth factors in culture supernatants of phytohemagglutinin (PHA)-stimulated human T lymphocytes (crude human T-cell growth factor from Associated Biomedic System, Buffalo, NY) were concentrated by precipitation with ammonium sulfate at 85% saturation, and an IL-2-enriched fraction was obtained from the precipitate fraction by chromatography on DEAE-cellulose (14). The mononuclear cells (10^6 nucleated cells per ml) were suspended in IL-2-containing medium and cultured with 2 μg of PHA-P (E-Y Laboratories, San Mateo, CA) per ml. After 4 days of culture, cells proliferating in the culture were resuspended in fresh IL-2-containing medium (2×10^5 cells per ml) and cultured in the absence of PHA.

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Abbreviations: IL-2, interleukin 2; Con A, concanavalin A; PNA, peanut agglutinin; PHA, phytohemagglutinin; E'-IgE, fixed ox erythrocytes coated with IgE; HAT, hypoxanthine/aminopterin/thymidine.

The RPMI 1640 culture medium described above was employed for the culture of RPMI 8866 lymphoblastoid cells and for the formation of IgE-binding factors. Human hybridoma cells were suspended in the culture medium at a concentration of 10^6 cells per ml, and the cell suspension was incubated for 24 hr in the presence of human IgE. In some experiments, the cells were cultured in IgE-coated well. A solution of human IgE in Dulbecco's phosphate-buffered saline (D-P_i/NaCl) (100 μg/ml) was placed in 2-ml Linbro wells. After overnight at 4°C the solution was removed and the wells were washed thoroughly with Hanks' balanced solution (BS solution). Hybridoma cells were placed in the wells and cultured for 24 hr.

Detection and Fractionation of IgE-Binding Factors. IgE-binding factors were detected by their ability to inhibit rosette formation of RPMI 8866 cells with fixed ox erythrocytes coated with human IgE (E'-IgE) (4). To increase the sensitivity of the assay for rosette inhibition, E'-IgE were prepared by sensitizing fixed ox erythrocytes with 0.02 mg of human IgE per ml (4). With these indicator cells, 25–45% of RPMI 8866 cells formed rosettes. Culture supernatants were filtered through Diaflo CF 50A membranes (cutoff point, M_r 50,000; Amicon) by a Centriflo system (15). E'-IgE were preincubated with a culture filtrate to be tested, and RPMI 8866 cells were added to the suspension for rosette formation (4). The percentage of rosette inhibition was determined in duplicate slides and was expressed as the average.

The IgE-binding factors in culture filtrates were absorbed with Ig-coupled Sepharose-CL by procedures previously described (16). One-milliliter aliquots of a 10-fold concentrated culture filtrate were absorbed by mixing with 0.25 ml of Ig-coated Sepharose for 90 min. The initial effluent and fluid washed with 3 ml of D-P_i/NaCl were combined. The beads were then eluted with 4 ml of glycine·HCl buffer (pH 3.0). Effluent and eluate fractions were dialyzed against D-P_i/NaCl.

Procedures for the fractionation of IgE-binding factors on lectin-coupled Sepharose have been described (16). Con A-Sepharose and lentil lectin-Sepharose were purchased from Pharmacia. Agarose coupled with PNA (PNA-agarose) was purchased from Sigma. Culture filtrates were concentrated 10-fold, and 1 ml of the concentrated material in D-P_i/NaCl was incubated with 0.3–0.5 ml of lectin-coupled Sepharose. The effluent and the fluid obtained by washing with 3 ml of D-P_i/NaCl were pooled. Con A-Sepharose and lentil lectin-Sepharose were then eluted with 4 ml of 0.2 M α-methylmannoside, while PNA-Sepharose was eluted with 0.2 M D-galactose (3).

The molecular size of IgE-binding factors was estimated by gel filtration through a Sephadex G-75 column (1.0 × 90 cm) that had been calibrated with ovalbumin (M_r 43,000), α-chymotrypsinogen (M_r 25,000), and ribonuclease (M_r 13,700) (16). Culture filtrates were applied to the column together with ^{125}I-labeled α-chymotrypsinogen, and 3-ml fractions were collected.

Detection of the Binding of ^{125}I-Labeled IgE (^{125}I-IgE) to Lymphocytes. Binding of ^{125}I-IgE dimer to hybridoma cells was determined by using Millititer 96-well filtration plates (5-μm pore size, Millipore). Fifty-microliter aliquots of a hybridoma cell suspension (4 × 10^6 cells per ml) was mixed with 100 μl (200 μg/ml) of either unlabeled human IgE or human IgG or Hanks' BS solution, and the cell suspensions were incubated for 45 min. Fifty microliters of human ^{125}I-IgE dimer (400 ng/ml) was then added to each well, and the suspensions were kept at room temperature for 2 hr. The cells were washed 5–10 times with Hank's BS solution, and the radioactivity of cells retained on the membranes at the bottom of the wells was determined in an automatic gamma counter.

RESULTS

T-Cell Hybridomas Secreting IgE-Binding Factors. The mononuclear cells from normal peripheral blood were cultured in IL-2-containing medium. T cells proliferating in the culture were resuspended in fresh medium (1 × 10^7 cells per ml) and cultured for 24 hr with 10 μg of human IgE per ml. The culture supernatant was filtered through a CF 50A membrane, concentrated 8-fold, and assessed for the presence of IgE-binding factors. As expected, IgE-binding factors were detected in the filtrate. T cells exposed to IgE were fused with BUC cells, and hybridomas developed in HAT medium were incubated overnight with 10 μg of human IgE per ml. Culture filtrates were assessed for the ability to inhibit rosette formation of RPMI 8866 cells with E'-IgE. Among 70 clones tested, the culture filtrates of 12 clones inhibited rosette formation by 22–65%. The culture filtrates of each clone were absorbed with human IgE-Sepharose and the beads were eluted in acid pH. The distribution of rosette-inhibiting activities in the effluent and eluate fractions showed that the rosette-inhibiting factor from 5 hybrid clones was absorbed with IgE-Sepharose and recovered in the eluates (Table 1). Aliquots of the same culture filtrates from the 5 clones were absorbed with either IgG-Sepharose or rat IgE-Sepharose. It is apparent in Table 1 that rosette-inhibiting factors did not have affinity for human IgG but were partially absorbed with rat IgE Sepharose. Approximately one-half of the factors was recovered from rat IgE-Sepharose by elution at acid pH.

To determine whether exposure of the hybridomas to IgE was required for the formation of IgE-binding factors, the five hybridomas described above were incubated for 24 hr in the presence or absence of 10 μg of human IgE per ml, and their culture filtrates were assessed for IgE-binding factors. As shown in Table 2, all of the hybridomas produced IgE-binding factors upon incubation with IgE. However, in the absence of IgE, the same cells failed to produce a detectable amount of IgE-binding factors. Experiments were then carried out to determine whether a short period of incubation with IgE was sufficient for the induction of IgE-binding factor formation. Human hybridomas were incubated with 10 μg of IgE per ml for 1 hr. After the culture supernatants were recovered, the cells were washed, resuspended in fresh medium, and cultured for 24 hr. IgE-binding factors were not detectable in 1-hr culture filtrates. However, filtrates

Table 1. Binding of rosette-inhibiting factor to IgE-Sepharose

Hybridoma	Human IgE-Sepharose (effluent/eluate), %	Human IgG-Sepharose (effluent/eluate), %	Rat IgE-Sepharose (effluent/eluate), %
166G11	1/29	34/0	18/24
166A2	3/37	36/4	15/31
165G2	3/40	34/0	26/24
166H3	0/34	28/4	20/21
170A3	3/32	33/5	23/28

Culture filtrates of hybridomas were absorbed with human IgE-Sepharose, human IgG-Sepharose, or rat IgE-Sepharose, and the beads were eluted at acid pH. IgE-binding factors in the effluent and eluate fractions were assessed by rosette inhibition. Values represent the % rosette inhibition.

Table 2. Requirement of IgE for the formation of IgE-binding factors

Hybridoma	Rosette-inhibiting activity in culture filtrate, %			
	IgE (−) (24 hr)	IgE (+) (24 hr)	IgE (+) (1 hr)	Second culture (24 hr)*
166G11	8	38	0	25
166A2	0	44	0	23
165G2	8	27	0	32
166H3	3	36	0	31
170A3	0	22	0	20

Hybridomas were cultured with or without 10 μg of human IgE per ml, and the culture filtrates were assessed for the presence of IgE-binding factors. Values represent the % rosette inhibition.
*Cells exposed for 1 hr to IgE were washed, resuspended in fresh culture medium, and cultured for 24 hr in the absence of IgE.

obtained from the second cultures contained substantial amounts of IgE-binding factors (Table 2).

We wondered whether polymerized IgE might be more effective than monomeric IgE for the induction of IgE-binding factor formation. Two representative hybridomas—i.e., 166G11 and 166A2—were subcloned, and the experiments were carried out with their subclones. Aliquots of cell suspensions were incubated for 24 hr with serial 1:10 dilutions of either a polymer, dimer, or monomer of human IgE, and IgE-binding factors in their culture filtrates were assessed. The minimal concentration of monomeric IgE required for the induction of factor formation was between 1 and 10 μg/ml. In contrast, even 10 ng of dimeric IgE or polymeric IgE per ml was sufficient for induction of the factor formation. It was also found that the hybridoma cells produced IgE-binding factors when the cells were cultured for 24 hr in IgE-coated wells. Rosette-inhibiting activity of culture filtrates from the IgE-coated wells was comparable to those of the same cells incubated for 24 hr with 10 μg of monomeric IgE per ml.

Induction of IgE-binding factor formation by IgE suggested that the hybridomas may bear $Fc_\varepsilon R$. Unfortunately, all of the hybridomas formed nonspecific rosettes with fixed ox erythrocytes coated with human serum albumin. Thus, we determined direct binding of dimeric IgE to hybridomas. When 2×10^5 T-cell hybridomas or RPMI 8866 cells were incubated with 100 ng of ^{125}I-IgE dimer per ml, RPMI 8866 cells bound 750 pg of IgE. The amount of dimeric IgE bound to T-cell hybridomas 166A2 and 166G11 was <20 pg and was comparable to that bound to BUC cells. However, if the same hybridomas were cultured in IgE-coated wells for 24 hr and the cells were incubated with ^{125}I-IgE dimer, 76–80 pg of the protein was associated with 2×10^5 hybrid cells. The binding of ^{125}I-IgE to T-cell hybridomas was completely inhibited by preincubation of the cells with 100 μg of unlabeled IgE per ml but not by the same concentration of unlabeled human IgG (see *Materials and Methods*). The results indicate that the hybridoma cells cultured in IgE-coated wells bear $Fc_\varepsilon R$ on their surface.

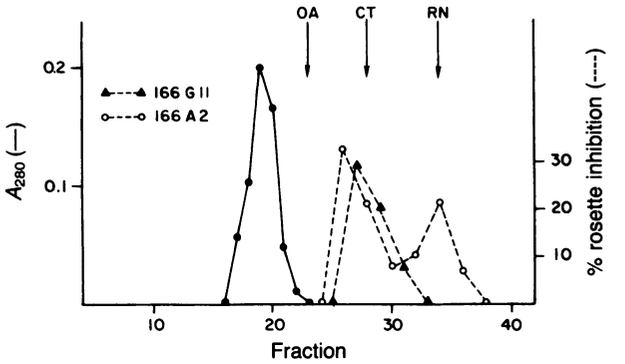

FIG. 1. Gel filtration of IgE-binding factors through a Sepharose G-75 column. Culture filtrates of 166A2/A3 and 166G11/D8 hybridomas were concentrated 50-fold and applied to a Sephadex column. Fetal calf serum (0.1 ml) was added to mark the void volume of the column. OA, CT, and RN represent elution volumes of ovalbumin, α-chymotrypsinogen, and ribonuclease from the column. The distribution of IgE-binding factors in eluate fractions was determined by rosette inhibition.

Physicochemical Properties of IgE-Binding Factors. The affinity of human IgE-binding factors for Con A, lentil lectin, or PNA was assessed. The results, summarized in Table 3, show that the majority of IgE-binding factors formed by all of the hybridomas bind to Con A-Sepharose and are recovered by elution with α-methylmannoside, but the factors fail to bind to PNA-agarose. It was also found that the majority of IgE-binding factors from two hybridomas—i.e., 166G11 and 166H3—bound to lentil lectin, whereas those from the other three hybridomas failed to bind to the lectin.

The molecular weight of IgE-binding factors was estimated by ultrafiltration through Diaflo membranes. Each hybridoma was cultured for 24 hr in the presence of 10 μg of IgE per ml, and culture supernatants were filtered through CF 50A or CF 25A membranes. As expected, the filtrates through CF 50A membranes inhibited rosette formation of RPMI 8866 cells by 30–50%. When CF 25A membranes were employed, the filtrates of 166A2 culture supernatants contained a substantial amount of IgE-binding factors. In contrast, the culture filtrates of the other four hybridomas did not contain a detectable amount of IgE-binding factors. Thus, we estimated the molecular weight of IgE-binding factors by gel filtration through a Sephadex G-75 column. Both 166G11/D8 and 166A2/A3 clones were cultured for 24 hr with IgE. The culture supernatants were passed through XM 50 membranes, and the filtrates were concentrated to 1/50th of the original volume of the culture supernatant. The concentrated filtrates were then applied to a Sephadex G-75 column, and eluate fractions were assessed for the presence of IgE-binding factors. As shown in Fig. 1, IgE-binding factors from 166G11/D8 cells gave a single peak that was eluted slightly earlier than α-chymotrypsinogen. In contrast, the factors from 166A2/A3 cells were composed of two species

Table 3. Affinity of IgE-binding factors for lectins

Hybridoma	Con A-Sepharose (effluent/eluate), %	PNA-agarose (effluent/eluate), %	Lentil lectin-Sepharose (effluent/eluate), %
166G11	0/31	30/3	10/24
166A2	0/27	26/3	29/6
165G2	0/27	27/4	20/5
166H3	0/21	27/0	4/21
170A3	0/24	27/4	20/6

Culture filtrates of each hybridoma were absorbed with Con A-Sepharose, PNA-agarose, or lentil lectin-Sepharose. The factors bound to the beads were eluted with either α-methylmannoside or D-galactose. IgE-binding factors in the effluent and eluate fractions were assessed by rosette-inhibiting activity. Values represent the % of rosette inhibition.

with M_rs of ≈30,000 and ≈15,000, respectively. The affinity of each species of IgE-binding factors for lectins was confirmed. Both the M_r 30,000 and M_r 15,000 IgE-binding factors from the 166A2/A3 clone bound to Con A-Sepharose, whereas the M_r 30,000 IgE-binding factor from the 166G11/D8 clone bound to lentil lectin-Sepharose.

DISCUSSION

Human hybridomas described in this report produced soluble factors that have affinity for human IgE but not for human IgG. Under the experimental conditions employed, approximately one-half of the factors formed by the hybridomas bound to rat IgE-Sepharose, suggesting that the factors have some affinity for rat IgE. However, the higher affinity of the factors for human IgE than rat IgE indicates that the factors are human IgE-binding factors. It was found previously (13) that rat IgE-binding factors bound to rat IgE but not to human IgE. The binding of human IgE-binding factors to rat IgE is not surprising. In rodent systems, it has been shown that IgE-binding factors are related to $Fc_\varepsilon R$ on lymphocytes (17). Although the binding of rodent IgE to $Fc_\varepsilon R$ on human lymphocytes has not been established, $Fc_\alpha R$ on human lymphocytes bind mouse IgA (18). It is also known that $Fc_\varepsilon R$ on human mast cells and basophils bind both human IgE and rodent IgE (19, 20), whereas human IgE does not bind to $Fc_\varepsilon R$ on rat mast cells.

A unique feature of human hybridomas described in this paper is that the cells are induced to form IgE-binding factors by stimulation with a specific ligand. Incubation of the hybridomas with 10 μg of human IgE per ml for 1 hr is sufficient to induce the cells to form the factors. The results are in agreement with the fact that rat T-cell hybridomas responded to rat IgE by forming IgE-binding factors (13). The present experiments also show that dimeric IgE is 1000-fold more effective than monomeric IgE for the induction of factor formation. Because polymerized IgE binds to $Fc_\varepsilon R$ much more avidly than monomeric IgE (11), the high efficiency of the polymerized IgE for factor formation may be explained by the effective binding of the protein to the lymphocytes. However, it is possible that induction of factor formation by "monomeric IgE" is due to the presence of polymerized IgE in the preparation and that monomeric IgE cannot induce factor formation. Although the monomer fraction did not contain a detectable amount of polymerized IgE, it is hard to exclude the possibility that 1% or less of the total protein in the preparation was polymerized. In any event, the induction of factor formation by as little as 10 ng of IgE dimer per ml suggests that soluble antigen–IgE antibody complexes may be involved in the formation of IgE-binding factors *in vivo*.

Induction of human IgE-binding factors by IgE strongly suggests that the hybridomas bear $Fc_\varepsilon R$. Although the receptors were not detected on unstimulated hybridomas, the same cells preincubated with IgE specifically bound a significant amount of IgE dimer. Previous studies have shown that incubation of normal rat mesenteric lymph node cells with rat IgE resulted in an increase in $Fc_\varepsilon R^+$ lymphocytes (21). One may speculate that the human T-cell hybridomas bear a small number of $Fc_\varepsilon R$, which are not sufficient for binding a detectable amount of IgE dimer, and that the binding of IgE to the receptors induced the expression of more $Fc_\varepsilon R$ and the formation of IgE-binding factors.

None of the five hybridomas described in this paper was stained by either OKT 3 or OKT 11 monoclonal antibody. However, the hybridomas appear to be derived from T cells. In the lymphocyte suspension fused to BUC cells, neither sIg^+ cells nor OKM 1^+ cells were detected. Approximately 70% of the cells were OKT 3^+ and 95% of the cells formed rosettes with neuraminidase-treated sheep erythrocytes (4). Experiments in which T-cell subsets were depleted before exposure to IgE indicate that the cell source of IgE-binding factor in the preparation is OKT 4^+ cells (results not shown).

IgE-binding factors formed by most of the human T-cell hybridomas had a M_r of >25,000, but one hybridoma, 166A2, produced two species—i.e., M_r 30,000 and M_r 15,000 IgE-binding factors. Their molecular sizes are comparable to those formed by rat lymphocytes (2, 13). Previous studies on rat IgE-binding factors indicated that IgE-potentiating factor contained N-linked, mannose-rich oligosaccharide and had affinity for both Con A and lentil lectin, whereas IgE-suppressive factor did not (3). It was also found that both factors had a M_r of 13,000–15,000 (2, 3). We have also demonstrated M_r 30,000 rat IgE-binding factor having neither potentiating activity nor suppressive activity (2). All human IgE-binding factors obtained in the present study had affinity for Con A, suggesting that the factors contain N-linked, mannose-rich oligosaccharides. Thus, the carbohydrate moieties in human IgE-binding factors are similar to those of rat IgE-potentiating factor. Because human IgE-binding factors have some affinity for rat IgE, we have recently assessed the effect of purified human IgE-binding factors on the IgE-forming cell response of rat mesenteric lymph node cells. The results indicated that purified IgE-binding factor from 166A2 hybridoma cells selectively potentiated the IgE response. Saryan *et al.* (22) cultured peripheral blood T cells of patients with hyper-IgE syndrome and obtained a soluble factor that selectively enhances the formation of IgE by normal human lymphocytes. The factor is a glycoprotein and appears to contain N-linked oligosaccharides. Their more recent experiments indicated that the soluble factor has affinity for human IgE (R. S. Geha, personal communication). We suspect that some of the IgE-binding factors formed by our T-cell hybridomas may be human IgE-potentiating factor.

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