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Prospective screening by a panfungal polymerase chain reaction assay in patients at risk for fungal infections: implications for the management of febrile neutropenia

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Summary. Invasive fungal infections are a major cause of mortality in neutropenic cancer patients. To determine whether a polymerase chain reaction (PCR)-based assay enabled the identification of patients at risk for invasive fungal infections, a prospective monitoring once per week was performed during 92 neutropenic episodes in patients receiving chemotherapy for acute leukaemia or high-dose therapy followed by allogeneic or autologous stem cell transplantation, with the investigators blinded to clinical and microbiological data. PCR positivity was documented in 34 out of 92 risk episodes. All patients developing proven invasive fungal infection were found PCR positive, and PCR was found to be the earliest indicator of invasive fungal infection preceding clinical evidence by a mean of 5·75 d (range 0–14 d). In febrile neutropenic patients without a prior history of invasive fungal infection, a sensitivity of 100% and a specificity of 73% of the PCR assay for the development of proven or probable invasive fungal infection was documented. In conclusion, panfungal PCR performed prospectively once a week enabled the identification of patients at high risk for invasive fungal infections.

Keywords: invasive fungal infection, early diagnosis, panfungal PCR, febrile neutropenia.

Invasive fungal infections have emerged as a major cause of morbidity and mortality in neutropenic cancer patients and in recipients of an allogeneic stem cell transplant (Horn *et al*, 1985; Whimbey *et al*, 1987; Denning, 1996; Wald *et al*, 1997). Early initiation of antifungal therapy is critical in reducing the high mortality rate in these patients (Aisner *et al*, 1977; Meyers, 1990; Fraser *et al*, 1992). However, as early diagnosis of fungal infections is difficult, a delay of antifungal therapy may be associated with increased morbidity and mortality. Given the rapidly fatal outcome of invasive fungal infections, early and accurate identification of fungal pathogens are a critical issue for proper targeting of antifungal therapy (Shin *et al*, 1997).

Polymerase chain reaction (PCR)-based detection systems have been developed that enable the amplification of a broad range of fungal pathogens within a single test tube (Niesters *et al*, 1993; Makimura *et al*, 1994; Haynes *et al*, 1995; Einsele *et al*, 1997; Van Burik *et al*, 1998). In highly selected groups of patients, PCR showed a promising sensitivity and specificity (Einsele *et al*, 1997; Van Burik *et al*, 1998). However, PCR for early diagnosis of fungal infections has not yet been assessed in unselected neutropenic cancer patients.

Therefore, a prospective panfungal PCR screening programme was performed in an unselected cohort of patients undergoing induction/consolidation chemotherapy for acute leukaemia or high-dose therapy followed by stem cell transplantation. PCR results were retrospectively correlated with the clinical course of the patients to determine whether the panfungal PCR test under study enabled the identification of patients at high risk for the subsequent onset of an invasive fungal infection, especially in the clinical situation of neutropenic fever of unknown origin.

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MATERIALS AND METHODS

Patients. Ninety-two risk episodes were analysed. Written

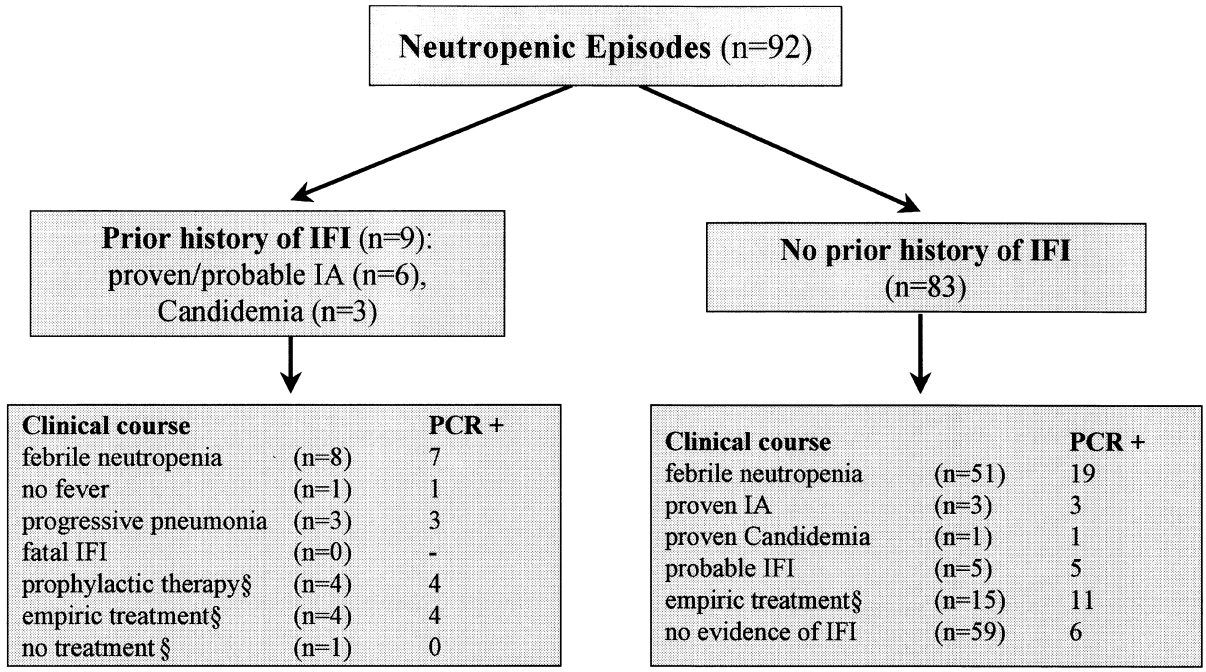

Fig 1. Clinical course of patients under study. IFI, invasive fungal infection; IA, invasive aspergillosis. §Application of intravenous antifungal therapy with amphotericin B.

informed consent was obtained from all patients under the guidelines of the institutional review board at the University Hospital of Tübingen.

Risk episodes were defined as the early post-transplant episode until day 30 in patients after stem cell transplantation (SCT) (autologous SCT, $n = 27$; allogeneic SCT, $n = 33$) as well as neutropenic episodes ($n = 32$) after induction/consolidation chemotherapy in patients suffering from acute lymphoblastic leukaemia ($n = 8$), acute myelogenous leukaemia ($n = 22$), myelodysplastic syndrome ($n = 1$) and Hodgkin's disease ($n = 1$). The mean age of patients under study was 37·8 (range 1–64) years. The median duration of neutrophils $< 0{\cdot}5 \times 10^9$/l in the transplant group was 13·9 d (range 6–47 d) and in the chemotherapy group was 14·2 d (range 4–36 d). A previous history of invasive fungal infection [proven or probable invasive aspergillosis (IA), $n = 6$; culture proven candidaemia, $n = 3$] was documented in nine neutropenic episodes analysed a median of 3 months (range 1–17 months) before enrolment on the study. The clinical course of the patients with respect to potential clinical signs of an invasive fungal infection (IFI) is shown in Fig 1.

Diagnosis of invasive fungal infection. For the diagnosis of a proven invasive fungal infection, either a tissue biopsy from a clinically documented lesion had to be positive for a fungal pathogen by histology and culture or at least one blood culture for *Candida* species in a febrile patient (candidaemia).

A probable invasive aspergillosis was defined as radiographic evidence compatible with *Aspergillus* infection and identification of *Aspergillus* species from sputum or bronchoalveolar lavage samples. In addition, a diagnosis of a probable invasive fungal infection was made in patients with febrile neutropenia and documentation of an air crescent or halo sign by computerized tomography (CT).

Anti-infectious prophylaxis. Antifungal prophylaxis was performed according to local protocols. All recipients of an allogeneic stem cell transplant were treated in laminar air flow rooms until recovery of neutrophils above $1{\cdot}0 \times 10^9$/l was documented. Antifungal prophylaxis in recipients of an allogeneic stem cell transplant consisted of fluconazole (400 mg/d) in 23 patients and low-dose intravenous amphotericin B (0·25 mg/kg body weight) in one patient. Nine patients received non-absorbable oral amphotericin B solution only.

Patients treated with high-dose chemotherapy followed by autologous SCT or chemotherapy only were nursed in rooms without laminar airflow. Systemic antifungal prophylaxis was applied in nine out of 30 recipients of an autologous stem cell transplant (fluconazole, $n = 4$; itraconazole, $n = 5$), and in 16 out of 32 risk episodes in patients receiving induction/consolidation chemotherapy (fluconazole, $n = 13$; itraconazole, $n = 2$, amphotericin B, $n = 1$).

Management of febrile neutropenia. Broad spectrum intravenous antibacterial therapy was initiated at the onset of fever, when the neutrophil count was below $0{\cdot}5 \times 10^9$/l. Fever was defined as a single axillary temperature of 38·5°C or at least two readings of > 38°C taken 2 h apart. Therapy was started after a clinical examination of the oral cavity, the skin and the perianal region was completed and a chest radiograph had been made. Blood was cultured from peripheral veins and a central venous catheter when present. Oropharyngeal, perianal, vaginal and skin swabs as well as stool and urine specimens were analysed for fungal growth. Routine screening for galactomannan in

Table I. Correlation of PCR results with clinical findings.

	Clinical course					
PCR results	All episodes ($n = 92$)	Previous history of IFI	*De novo* proven IFI	Probable IFI	Empirical amphotericin B	No IFI
PCR positive for						
Aspergillus spp.	17	4 (IA)	2 (IA)	5	5	1
Aspergillus spp. + *C. albicans*	4	1 (IA)	1 (IA)	–	2	–
C. albicans	8	–	–	–	4	4
C. glabrata	5	3 (candidaemia)	1	–	–	1
PCR negative	58	1	–	–	4	53
	Clinical course					
PCR results	Febrile neutropenia ($n = 51$)	Previous history of IFI	*De novo* proven IFI	Probable IFI	Empirical amphotericin B	No IFI
PCR positive for						
Aspergillus spp.	13	–	2 (IA)	5	5	1
C. albicans	6	–	–	–	5	1
PCR negative	32	–	–	–	4	28

IA, invasive *Aspergillosis*; IFI, invasive fungal infection.

serum samples was not performed, and positive results if available were not considered for a diagnosis of invasive aspergillosis. CT of the lungs was performed in patients with a suspected fungal infection, and bronchoalveolar lavage samples were taken in patients with pulmonary infiltrates only.

Empirical systemic therapy with amphotericin B at a dosage of 0·6–1·0 mg/kg body weight was initiated in patients with neutrophil counts below 1×10^9/l who remained febrile after 96 h on broad spectrum antibiotics. Patients with pulmonary infiltrates suggestive of invasive pulmonary aspergillosis with or without microscopic or cultural detection of *Aspergillus* species received intravenous amphotericin B at a dosage of 1·0–1·5 mg/kg body weight.

Collection and handling of blood specimens. Ethylene diamine tetra-acetic acid (EDTA)-anticoagulated whole blood specimens (5 ml) were collected prospectively once per week starting at admission to the hospital. DNA extraction of samples was performed as described before (Einsele *et al*, 1997). The PCR assay was run by an investigator blinded to clinical and microbiological data. PCR results were not known to the physicians and thus not used in the management of the patients. Fungal DNA extraction, PCR amplification and hybridization with *Aspergillus*- and *Candida*-specific probes were performed in Tübingen. After demonstrating that the sensitivity of the PCR assay performed on EDTA-anticoagulated blood samples spiked with fungal cells and DNA and stored for up to 72 h was not affected (data not shown), samples from Ulm and Frankfurt were shipped overnight at room temperature to Tübingen and extracted immediately after arrival. Thus, DNA extraction from all clinical samples was performed within 24 h after collection of blood samples.

PCR assay and hybridization. The oligonucleotide primer pair (5′-ATTGGAGGGCAAGTCTGGTG, 5′-CCGATCCCTAGTCGGCATAG) and the DNA probes specific for *Aspergillus* species (CATGGCCTTCACTGGCTGTGGGGGGAACCA), *Candida albicans* (TCTGGGTAGCCATTTATGGCGAACCAGGAC), *Candida glabrata* (TTCTGGCTAACCCCAAGTCCTTGTGGCTTG), *Candida krusei* (GTCTTTCCTTCTGGCTAGCCTCGGGCGAAC), *Candida tropicalis* (GTTGGCCGGTCCATCTTTCTGATGCGTACT) and *Candida parapsilosis* (TTTCCTTCTGGCTAGCCTTTTTGGCGAACC) have been described previously (Einsele *et al*, 1997). Thirty-five cycles of repeated denaturation, primer annealing and enzymatic chain extension (30 s at 94°C, 1 min at 62°C, 2 min at 72°C) were performed in a Biometra Trioblock thermocycler (Biometra, Göttingen, Germany). A maximum of 24 samples including controls were run in each PCR assay. To monitor for contamination, aliquots of saline and human fibroblast DNA were prepared concurrently as negative extraction and amplification controls by the same procedure. For each 10 blood samples extracted or amplified, one of each type of negative control was added according to the recommendations of the German Society for Microbiology. Blood samples spiked with different amounts of *Aspergillus fumigatus* and *C. albicans* cells were extracted concurrently to control the DNA extraction step. To control the sensitivity of the assay, fungal DNA was amplified in each run in concentrations ranging from 100 fg to 100 pg. Co-amplification of the HLA-DR gene was performed in each test tube to exclude the presence of *Taq* inhibitors. Aliquots (10 μl) of each amplification product were electrophoretically separated in a 2% agarose gel in 1× Tris-acetate–EDTA (TAE) buffer, pH 8·0 (40 mmol/l Tris-acetate, pH 7·5, 2 mmol/l sodium EDTA) followed by ethidium bromide staining. The amplicons were analysed by a slot-blot test with 5′-labelled internal

oligonucleotide probes. Hybrids were incubated with Anti-Digoxigenin-Alkaline Phosphatase (150 U/l Fab fragments, Boehringer Mannheim, Germany) for 20 min and visualized with nitroblue tetrazolium and bromochlorindoylphosphatetoluidine as described previously (Einsele *et al*, 1997).

RESULTS

PCR screening

A total of 333 samples, a median of 3·6 per risk episode (range 2–7), were analysed, 242 (72·6%) of which tested negative and 91 positive (27·4%). PCR positivity was documented in 34 out of 92 (37%) episodes analysed. PCR revealed a positive result for *Aspergillus* species in 17, for *Aspergillus* species and *C. albicans* in four, for *C. albicans* in eight and for *C. glabrata* in five risk episodes. Six out of the 34 PCR positive patients (17·6%) neither developed clinical signs of invasive fungal infection nor received empirical antifungal treatment (false-positive test results) (Table I). Thus, only 6 out of 59 patients without clinical evidence of an invasive fungal infection (10·2%) were found to be PCR positive (Table I).

Correlation of the PCR results with clinical findings

Of the 17 patients tested positive for *Aspergillus* species, four had a previous history of IA, two developed proven and five probable IA and five received intravenous amphotericin B for persisting febrile neutropenia in spite of broad spectrum antibiotic treatment. Only 1 out of these 17 patients did not receive antifungal treatment.

Four patients were found to be PCR positive for *Aspergillus* species and *C. albicans*; one had a previous history of IA, one developed proven IA and two received intravenous amphotericin B for a fever not responding to broad spectrum antibiotic treatment. In addition, three of the four patients were colonized with *C. albicans* at three or more localizations.

Eight patients tested positive for *C. albicans*; four were treated with intravenous amphotericin B for febrile neutropenia and documentation of pulmonary infiltrates by chest radiographs and four patients had no clinical evidence of IFI, two out of whom tested positive only once, one twice and one patient three times. Six out of these eight patients PCR positive for *C. albicans* were colonized at three or more localizations by *C. albicans*.

Five patients were found to be PCR positive for *C. glabrata*; two had a previous history of candidaemia due to *Candida glabrata*, one patient subsequently developed culture-proven candidaemia and one patient, also with a previous history of candidaemia due to *Candida glabrata*, developed a pneumonia with a bronchoalveolar lavage showing no other pathogen explaining the pulmonary infiltrates. Resolution of pulmonary infiltrates was documented after the introduction of antifungal therapy with intravenous amphotericin B. One additional patient found to be PCR positive on two occasions did not show any clinical signs of invasive candidiasis (Table I).

All four patients developing proven invasive fungal infection during the screening programme were found to be PCR positive, and PCR was found to be the earliest indicator of invasive fungal infection preceding non-specific radiological findings in three patients with invasive aspergillosis by a mean of 1 d (range −2–5 d) and the first positive culture result in a patient with candidaemia by 14 d (Table II). The mean time from PCR positivity to the initiation of antifungal treatment was 5·75 d (range 0–14 d).

Patients with a positive PCR result at study entry

Eleven patients were found to be PCR positive at admission to the hospital when the neutrophil counts were still $> 0{\cdot}5 \times 10^9$/l, six for *Aspergillus* species, two for *C. albicans* and three for *C. glabrata*. Four of these 11 patients had a previous history of an invasive fungal infection (IA, $n = 2$;

Table II. Clinical characteristics of patients with *de novo* proven invasive fungal infection.

Age (years)	Sex	Diagnosis	Diagnosis of IFI	Time from positive PCR to radiology	Antifungal therapy	Outcome	Comments
43	M	CML, MUD-BMT	Post-mortem: *A. fumigatus* in lungs and liver	+5	+6	Death due to IA	–
68	M	AML, reinduction chemotherapy	BAL: *A. fumigatus*	+1	+3	Death due to IA	–
23	F	AML, induction chemotherapy	Blood culture: *C. glabrata*	–	+14	Survived	–
59	M	AML, reinduction chemotherapy	Lung biopsy: septate branched hyphae	0*	0	Survived	PCR+ for *A. fumigatus* 5 d before progressive pneumonia

*This patients was found to be PCR positive for *C. albicans* in repetitive samples and developed a positive PCR result for *Aspergillus* DNA 5 d before progressive pneumonia, 2 d after the first documentation of a non-specific pulmonary infiltrate on the chest radiograph.CML, chronic myeloid leukaemia; AML, acute myeloid leukaemia; MUD, matched-unrelated donor; BMT, bone marrow transplantation; IFI, invasive fungal infection; BAL, bronchoalveolar lavage, IA, invasive *Aspergillosis*

culture-proven *C. glabrata* blood stream infection, $n = 2$), four developed an invasive fungal infection during the subsequent risk episode (proven IA, $n = 1$; probable IFI, $n = 2$; culture-proven *C. glabrata* blood stream infection, $n = 1$) and two patients received empirical antifungal therapy for febrile neutropenia not responding to broad spectrum antibiotic therapy after 96 h. Only one patient positive for *C. albicans* did not receive antifungal treatment during the subsequent neutropenic episode.

PCR in febrile neutropenic patients without a history of an invasive fungal infection

Fifty-one out of 81 (64·2%) patients without a previous history of invasive fungal infection developed febrile neutropenia, 16 after induction/consolidation chemotherapy and 35 after stem cell transplantation. A PCR assay performed within 72 h after onset of febrile neutropenia showed 19 patients to be PCR positive (*Aspergillus* DNA, 13; *C. albicans* DNA, six). Six out of these 51 patients were already found to be PCR positive at study entry.

Two out of these 19 patients developed proven and five patients probable IFI, 10 patients received empirical antifungal therapy for persisting fever in spite of broad spectrum antibiotic therapy and only two patients, one positive for *C. albicans* and one positive for *Aspergillus* species, did not receive antifungal treatment. None of the 32 PCR-negative patients with febrile neutropenia developed a proven or probable invasive fungal infection, and only 4 of these 32 patients received empirical antifungal treatment for febrile neutropenia not responding to broad spectrum antibiotic therapy after 96 h (Table I).

Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of a single PCR assay performed at the onset of febrile neutropenia were calculated. For subsequent development of a proven or probable invasive fungal infection, the sensitivity was found to be 100%, the specificity 73%, the PPV 36·8% and the NPV 100%.

Potential benefit of PCR screening to monitor the efficacy of antifungal therapy

In 23 out of the 34 PCR-positive neutropenic episodes, sufficient samples were available to assess the potential benefit of a continued PCR screening during follow-up. In 11 patients, clearance of the fungal DNA at the end of the study was documented, 10 out of whom cleared the fungal DNA during antifungal therapy and one patient at the time of leucocyte recovery. None of these patients developed an invasive fungal infection during follow-up. Twelve patients were found to be persistently PCR positive, three of whom died with invasive fungal infection and two developed an invasive fungal infection later. Five persistently PCR-positive patients had a previous history of an invasive fungal infection before the screening episode. In two patients, no correlation of the positive PCR results and the clinical course was seen.

DISCUSSION

Invasive fungal infections have become a major cause of infection-related morbidity and mortality in neutropenic cancer patients and recipients of an allogeneic stem cell transplant. Current limitations in the diagnosis of invasive fungal infections include the non-specific nature of clinical symptoms and the relative insensitivity and poor specificity of conventional microbiological methods. Given these limitations, empirical antifungal therapy for febrile neutropenia not responding to broad spectrum antibiotic therapy has become a standard therapy to treat early a possible, and to prevent a secondary invasive fungal infection associated with prolonged neutropenia (Nuzzi *et al*, 1997). In spite of these efforts, a prevalence of 15–20% for invasive aspergillosis and invasive candidiasis has been reported in patients at high risk, such as recipients of an allogeneic stem cell transplant and patients with prolonged neutropenia after induction/consolidation chemotherapy for acute myeloid leukaemia (Bow *et al*, 1995; Groll *et al*, 1996; Wald *et al*, 1997).

Here, we describe a prospective screening programme with a panfungal PCR assay in patients at risk for invasive fungal infections. PCR positivity was documented in 34 out of 92 risk episodes. In eight out of these 34 PCR-positive risk episodes, a previous history of an invasive fungal infection before study enrolment was documented, four patients developed a proven and five a probable invasive fungal infection, and 11 patients received empirical antifungal treatment for febrile neutropenia not responding to broad spectrum antibiotic treatment after 96 h. Thus, only six PCR-positive patients neither developed clinical signs of invasive fungal infection nor received empirical antifungal treatment. False-positive test results have been reported also for other sensitive assays (Swanink *et al*, 1997). Given the rapidly fatal outcome of proven invasive fungal infections, overtreatment to a certain extent seems acceptable. In addition, risk stratification based on clinical risk factors, results of sensitive screening assays as well as high-resolution CT scans performed during the early course of febrile neutropenia might help to reduce the number of patients treated with potentially toxic antifungals for persistent fever only (Severens *et al*, 1997).

At study entry, 11 patients were found to be PCR positive. Four of these patients had a prior history of invasive fungal infection, a group of patients likely to be colonized and to reactivate fungal infection during a subsequent neutropenic episode (Einsele *et al*, 1998; Offner *et al*, 1998). Four of the 11 patients developed proven or probable invasive fungal infection and two received empirical antifungal therapy for febrile neutropenia not responding to broad spectrum antibiotic therapy. Thus, 10 out of 11 patients presenting with a positive PCR at start of chemotherapy had evidence of an invasive fungal infection, indicating that PCR helps to identify patients at high risk for invasive fungal infections that might benefit from intensified prophylactic strategies.

In spite of significant toxicity, as many as 68% of neutropenic patients will receive conventional amphotericin B, most often given for persistent fever only (Goodman *et al*,

1992; Winston *et al*, 1993). We were thus interested to analyse whether PCR performed at the onset of febrile neutropenia might help to identify patients who are likely to develop invasive fungal infections. Fifty-one patients without a previous history of invasive fungal infection developed febrile neutropenia. Nineteen patients were found to be positive by PCR performed within 72 h after onset of fever, with 7 out of these 19 patients presenting with a proven or probable invasive fungal infection and another 10 patients receiving empirical antifungal treatment. In contrast, only 4 out of 32 PCR-negative patients received empirical antifungal therapy. The positive predictive value of a positive PCR performed at the onset of febrile neutropenia was found to be 36·8% for proven and probable invasive fungal infection, indicating that PCR performed at the onset of febrile neutropenia may enable the selection of patients who might benefit from immediate antifungal therapy. In addition, clearance of fungal DNA from the blood was associated with resolution of clinical symptoms, whereas patients with fatal invasive fungal infection showed persistence of fungal DNA in spite of antifungal therapy.

In conclusion, these data indicate that prospective screening with sensitive diagnostic techniques might have important implications for the future management of patients at risk for invasive fungal infections. Patient selection based on sensitive screening assays, radiology and clinical assessment might help to initiate early antifungal therapy in patients at high risk and to reduce overtreatment with potentially toxic and also expensive antifungal compounds in patients with a persistent fever only.

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