

Cultivation of Fungi in Synthetic and Semi-Synthetic Liquid Medium

II. Immunochemical Properties of the Antigenic and Allergenic Extracts

S. VAN DER HEIDE, H. F. KAUFFMAN and K. DE VRIES

Department of Allergology, Clinic for Internal Medicine, State University Hospital, Groningen, The Netherlands

Four allergologically important fungi, viz. *Aspergillus fumigatus*, *Penicillium notatum*, *Alternaria alternata*, and *Cladosporium herbarum* were cultured in a liquid synthetic medium, with or without addition of 0.1 % yeast extract (YE). After 10 and 28 days of cultivation, immunochemical properties of the fungal extracts, characterised by precipitation pattern and IgG- and IgE-binding capacity, were studied. In pure synthetic medium no large differences were observed in number of precipitates, as measured by DID, among the four antigenic fractions of each fungus (metabolic and mycelial antigens of both early and late phase cultures). Addition of YE to the growth medium hardly changed the number of precipitates and generally caused a decrease in IgG-binding capacity of the extracts. In contrast to these observations were the findings with the IgE-binding capacities of the fungal extracts as measured by RAST. Most allergenic fractions demonstrated an increase (sometimes strong) in IgE-binding capacity after YE was added to the growth medium. It is concluded that the time of cultivation influences the immunochemical characteristics of fungal extracts and that mycelial as well as metabolic antigens of both early and late phase cultures should be used in order to obtain a wide spectrum of allergens in extracts used for diagnostic purposes.

Key words: allergens; ELISA; fungi; precipitation; RAST; standardization.

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Standardization and characterization of allergenic extracts which are used for diagnostic purposes and immunotherapy is important and necessary (2, 16). Standardization of fungal allergenic extracts is hampered because of the great complexity of these extracts (1, 4) while allergenic extracts which are commercially available may vary strongly in allergenic potency and allergen content (12, 17). Knowledge of the culture conditions and growth characteristics of allergologically important fungi, viz. *Aspergillus fumigatus*, *Penicillium notatum*, *Alternaria alternata*, and *Cladosporium herbarum* is limited. Previous studies on *Aspergillus fumigatus* showed that one of the important variables in the antigenic composition of fungal culture filtrates is the time of cultivation and the composition of the culture medium (8, 10). In these studies

differences were shown between early and late phase antigenic extracts.

In an accompanying paper the growth characteristics of the above-mentioned four fungi, cultured in a synthetic medium, and the corresponding biochemical properties of the antigenic extracts obtained after different periods of cultivation, are shown (6). It was demonstrated that the time of cultivation strongly influences the metabolic states of the cultures, showing similarities for *A. fumigatus* and *P. notatum*, whereas *A. alternata* and *C. herbarum* showed different growth characteristics. In this paper the immunochemical properties of culture filtrate extracts and mycelial extracts of early phase and late phase cultures of these four fungi were investigated. Since the fungi showed a limited growth on pure synthe-

tic medium (6), the effect of enrichment of the synthetic medium with yeast extract on the immunochemical properties was additionally studied. The clinical relevance of these findings is discussed.

MATERIAL AND METHODS

Antigens

The four fungi: *A. fumigatus*, *P. notatum*, *A. alternata*, and *C. herbarum*, were cultured in liquid synthetic medium (Czapek-Dox broth) with and without 0.1% yeast extract (YE). After 10 days and 28 days of growth in submerged cultures at 27°C with constant agitation (120 rpm) flasks were harvested. Antigenic extracts were prepared from culture fluid (metabolic antigens) and from mycelium as described in the accompanying paper (6).

Rabbit antisera

Antisera were prepared by immunizing three rabbits for each fungus with an antigen mixture (10 mg antigen/0.5 ml Freund's adjuvant/injection) consisting of equal amounts (dry weight) of mycelial and metabolic antigens of both early and late phase cultures. Extracts were used from cultures *without* YE in the culture fluid. Injections were repeated every 10 days until double-immunodiffusion showed no further change in antibody titre.

Patient sera

A pool of serum was prepared from eight patient sera containing high titres of IgE and IgG antibodies against all four fungi.

A serum pool of 50 sera from normal individuals, obtained from the Blood Transfusion Service, was used as a negative control.

Crossed-immunoelectrophoresis (CIE) was carried out as described by Weeke (15), using 10% hyperimmune rabbit sera in the second dimension. Antigen (50 mg/ml), consisting of the same antigen mixture as used for immunization of the rabbits, was run in the first dimension.

Double-immunodiffusion (DID) was carried out according to Ouchterlony using antigen concentrations of 50 mg/ml and patient sera or rabbit hyperimmune sera as described by Kauffman et al. (9).

IgG-binding capacity of the different antigenic extracts was measured by the enzyme-linked immunosorbent assay (ELISA) as described previously (5). Briefly: Greiner AR microtiter plates were coated with antigen solution (1 mg/ml in 0.1 M NaHCO₃, pH 9.6). After washing with PBS (pH 7.4) plates were successively incubated for 1 h at 37°C with: 1) human sera (positive pool serum or negative control pool serum, both diluted 1:250), 2) rabbit anti-human IgG (Fc) (Nordic Immunology, Tilburg, The Netherlands) diluted 1:500, and 3) Alkaline Phosphatase Anti-Rabbit IgG (Goat) (Miles-Yeda Ltd, Israel), diluted 1:1000. Finally, the plates were incubated with substrate (p-nitrophenylphosphate) for 30 min at room temperature. The reaction was stopped with 50 µl 3 N NaOH, and after a nine-fold dilution of the samples, the absorbance was measured at 405 nm.

IgE-binding capacity of the different allergenic extracts was measured by RAST, using the Phadezym-RAST procedure (Pharmacia Diagnostics AB, Uppsala, Sweden). Paper discs were activated by cyanogen bromide (CNBr) as described by Ceska & Lundkvist (3). Allergenic extracts were coupled to CNBr-activated paper discs as described by Karr et al. (7), using allergen concentrations of 10 mg/ml and 10 discs/2.5 ml coupling buffer.

RESULTS

Precipitating properties

The precipitating properties of the four fungal extracts were studied by CIE, using rabbit hyperimmune sera (Fig. 1). About 24, 18, 22 and 29 precipitates could be detected with antigenic extracts of *A. fumigatus*, *A. alternata*, *P. notatum* and *C. herbarum*, respectively. Precipitat-

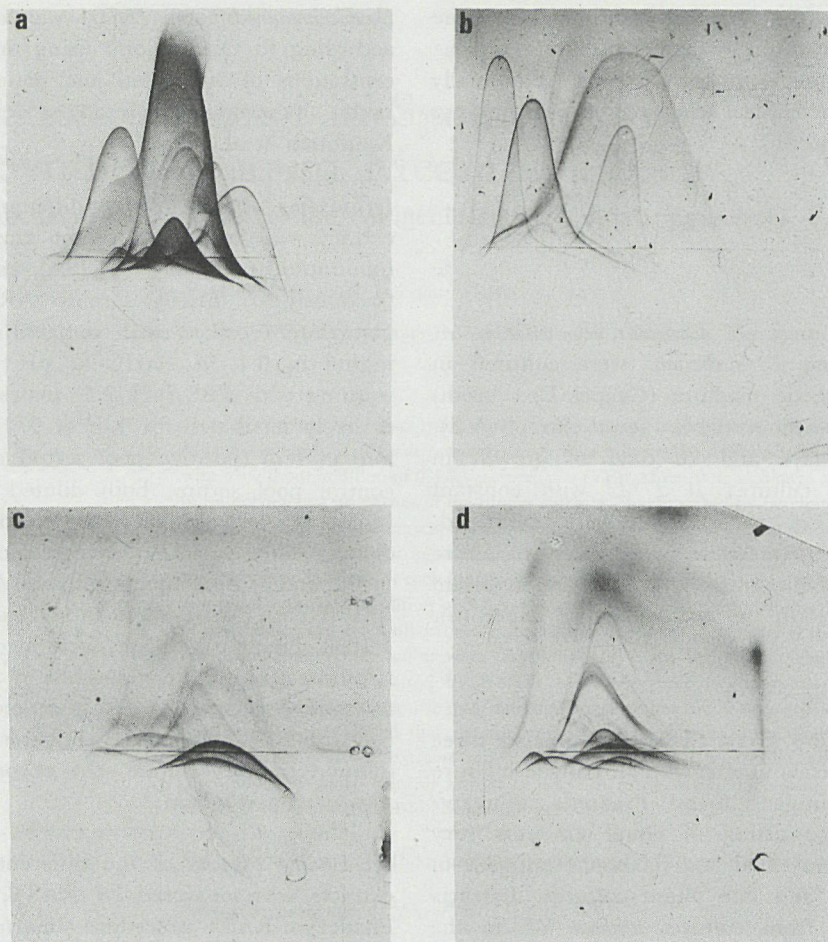


Fig. 1. CIE of four fungal extracts against the corresponding rabbit antisera. Each extract consisted of a mixture of metabolic and mycelial antigens of both early and late phase cultures (without yeast extract). a: *A. fumigatus*; b: *P. notatum*; c: *A. alternata*, and d: *C. herbarum*.

ing properties of the mycelial and culture fluid antigenic fractions of each fungus of both early and late phase cultures, and the effect of enrichment of the synthetic medium with 0.1 % YE on the number of precipitates were investigated by DID, using the same rabbit antisera (Table 1).

Without YE in the culture fluid, no large differences were shown in number of precipitates between early phase (e) and late phase (l) antigens or between metabolic (CF) and mycelial (M) antigens. An exception was found with the mycelial antigens of *A. alternata*, which demonstrated higher numbers of precipitates

when compared with the corresponding metabolic antigens.

Although the number of precipitates was rather similar for early and late phase cultures, there were differences in antigenic composition, as some precipitates of antigens from early phase cultures did not show lines of identity with antigens obtained from late phase cultures, and vice versa. Although addition of YE to the culture medium caused a marked increase in metabolism and growth of the fungi, no clear effect was found on the number of precipitates after YE was added to the growth medium. Six extracts (out of 16) showed an increase in

number of precipitates, five extracts a decrease, while in five antigenic extracts the number of precipitates was unchanged. It should be noted, however, that the rabbits have been immunized with antigens obtained from YE-free cultures. Therefore, it is possible that YE-enriched cultures contain antigens which are not recognized by the rabbit antisera. Influence of residual YE in the antigenic extract on the precipitation patterns was excluded since the rabbit antisera of rabbits that were sensitized by antigens from YE-deprived cultures did not show precipitates to YE itself (not shown).

DID studies were also carried out with nine sera of patients with *aspergillosis*, using late phase culture fluid antigen of *A. fumigatus* (from cultures with and without YE in the growth medium). After addition of YE, the number of precipitates rose from $4.2 (\pm 1.3 \text{ SD})$ to $5.8 (\pm 1.8)$. None of the individual patient sera showed precipitates to YE itself. Thus, addition of YE to the culture fluid caused a limited increase in number of precipitates as measured by DID. Similar studies were not possible for the other fungi due to the limitations of positive sera. It is important to note that after addition of YE to the growth medium a strong increase in the yield of antigenic material was observed, as shown in the accompanying paper (6, Table 3).

IgG-binding properties

A sensitive and more quantitative procedure for detecting the antibody binding capacity of fungal extracts is the enzyme-linked immunosorbent assay (ELISA). The IgG-binding capacity of the extracts was measured by

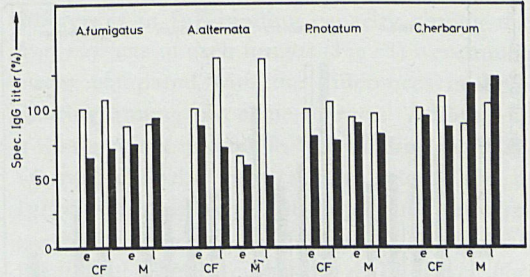


Fig. 2. IgG-binding capacities of the different fungal extracts of cultures without (□) and with (■) 0.1 % yeast extract (YE) in the culture fluid, as measured by ELISA.

The absorbance values at 405 nm found with the pool of patient sera were corrected for the A_{405} nm values found with a pool of normal sera. IgG titres are expressed as percentages, taking, arbitrarily, the absorbance value of the CF-e extract of each fungus of YE-free cultures as 100 %. CF: culture filtrate antigens; M: mycelial antigens; e: early phase culture (10 days); l: late phase culture (28 days).

ELISA, using a pool of patient sera and is summarized in Fig. 2.

Without YE in growth medium (open bars) the antigenic fractions from late phase cultures (l) showed a tendency to higher IgG-binding compared with early phase (e) antigens. The late phase metabolic antigen (CF-l) of all four fungi showed the highest IgG-binding. Most of the IgG-binding components, however, were already released in the culture fluid during the early phase of cultivation. The metabolic antigens (CF) of *A. fumigatus* and *P. notatum* demonstrated higher IgG-binding when compared with the corresponding mycelial antigens.

Addition of YE to the synthetic growth medium caused a decrease in the release of IgG-binding components in the culture fluid for all four fungi, during both the early and late

Table 1

Number of precipitation lines found in the DID with the different antigenic extracts and the corresponding rabbit antisera. Antigens were obtained from both culture fluid (CF) and mycelium (M) from early phase (e) and late phase (l) cultures. Cultures were made with or without yeast extract (YE) in the culture fluid

	<i>A. fumigatus</i>		<i>A. alternata</i>		<i>P. notatum</i>		<i>C. herbarum</i>	
	-YE	+YE	-YE	+YE	-YE	+YE	-YE	+YE
CF-e	9	11	6	5	5	5	4	4
CF-l	6	10	6	7	7	5	5	6
M-e	11	9	n.d.	11	7	7	6	5
M-l	9	11	10	10	6	7	6	4

n.d.: not determined.

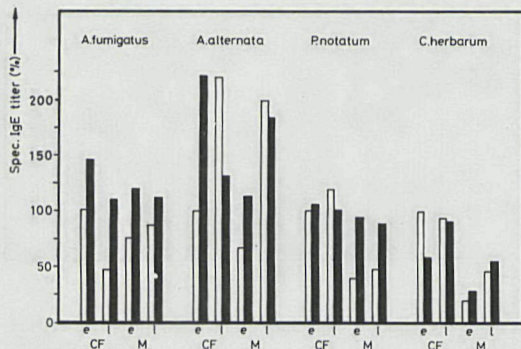


Fig. 3. IgE-binding capacities of the different fungal extracts of cultures without ($\square$) and with ($\blacksquare$) 0.1% yeast extract (YE) in the culture fluid, as measured by RAST. Details are as described under Fig. 2.

phase of cultivation (Fig. 2, closed bars). Also the IgG-binding capacity of the somatic antigens (M) was decreased for *A. alternata* and *P. notatum*. By contrast, the mycelial antigens of *C. herbarum* demonstrated an increase in IgG-binding, whereas the mycelial antigen of *A. fumigatus* showed a decline for early phase antigens and an increase for the late phase antigens. When YE is present in the growth medium, all mycelial antigens (except for *A. alternata*) now showed higher IgG-binding capacities compared with the corresponding metabolic antigens.

IgE-binding properties

The IgE-binding capacities of the allergenic extracts as measured by RAST were often in marked contrast to the IgG-binding characteristics both when studied after different periods of cultivation and after addition of YE to the culture medium (Fig. 3).

In YE-free cultures (open bars), large differences in IgE-binding were found between early and late phase metabolic allergens of *A. fumigatus* and *A. alternata* (CF). Relatively high IgE-binding was found for early phase metabolic allergen of *A. fumigatus* (CF-e) when compared with the corresponding late phase allergen (CF-l). By contrast, *A. alternata* showed the highest IgE-binding with the late phase metabolic allergen. The differences in IgE-

binding between early and late phase metabolic allergens of *P. notatum* and *C. herbarum* were less marked.

Differences in IgE-binding were also found between metabolic and mycelial allergens of both *P. notatum* and *C. herbarum*, showing higher IgE-binding potencies for the metabolic allergens.

In contrast to the IgG-binding characteristics, the IgE-binding capacities of the allergenic extracts were generally increased after addition of YE to the growth medium (closed bars). In YE-enriched cultures, the early phase metabolic allergens (CF-e) appear to be high IgE-binding; only the CF-e allergen of *C. herbarum* showed a fall in IgE-binding after addition of YE. The strongest effects of the addition of YE on IgE-binding were found with allergenic extracts of *A. fumigatus*, where all four extracts demonstrated an increase in IgE-binding after addition of YE. Also the CF-e extract of *A. alternata* showed a marked increase in IgE-binding. After addition of YE to the growth medium, the differences in IgE-binding between early and late phase allergens, or between metabolic and mycelial allergens, have become less pronounced when compared with YE-deprived cultures. When the IgE-binding capacity of allergenic extracts of YE-enriched cultures was measured, using five individual patient sera, it appeared that the IgE-antibody response to mycelial and metabolic allergens may vary strongly (not shown). Some sera showed preferential IgE-response to one or both mycelial extracts, whereas other patient sera demonstrated higher IgE-binding with metabolic allergens. Similar differences were observed between early and late phase allergens.

DISCUSSION

It has already been shown that differences in growth conditions of fungi may strongly influence the antigenic composition of fungal extracts (8, 13). In this study the immunochemical properties of fungal extracts obtained after 10 and 28 days of cultivation in a synthetic liquid medium were investigated.

It was shown that the number of precipitates found in the DID with rabbit antisera, did not differ much among the four extracts of each fungus (mycelial and metabolic antigens of both early and late phase cultures). Furthermore, the number of precipitates was not markedly changed by the addition of YE to the synthetic medium. In accordance with similar observation on *A. fumigatus* (11), it was shown that during longer cultivation new antigenic components are released. When nine individual patient sera were used in DID studies only a limited increase in precipitates of late phase metabolic antigens of *A. fumigatus* was found after addition of YE to the growth medium.

The IgG-binding capacities of the several antigenic extracts, as measured by ELISA using a pool of patient sera, differed from the results obtained by DID. Although IgG-binding values were rather similar for the four antigenic fractions of each fungus, as was also found by DID, the effect of the addition of YE to the culture fluid was quite different. Most extracts showed a decrease in IgG-binding, whereas in DID the number of precipitates did not change or showed a small increase after YE was added to the growth medium. Similar discrepancies between results from ELISA and DID have already been observed by Kauffman et al. (9) where it was shown that the amount of polystyrene binding components as measured by ELISA and number of precipitating components are not closely related. This discrepancy is ascribed to differences in antigenic components that are detected in both assays. Notwithstanding the decrease in IgG-binding components as found by ELISA, after addition of YE to the growth medium the antigenic yield of metabolic and mycelial material was largely enhanced, as shown in the accompanying paper. This suggests that relatively more non-IgG-binding components are synthesized after addition of YE.

The reverse was found for allergenic (IgE-binding) components, where generally an increase (sometimes even a twofold increase) was observed after addition of YE to the culture fluid. Without YE in the growth medium, the

differences in IgE-binding capacity among the four extracts of each fungus (Fig. 3) were much larger compared with the differences in IgG-binding among the same extracts. Addition of YE may result in a fall in IgG-binding capacity, whereas the same extracts may show a rise in IgE-binding capacity. This implies that extracts can be found with a low IgG-binding (e.g. CF-I fraction of YE-free culture of *A. fumigatus*, Fig. 2), but showing a high IgE-binding capacity. No relation was found between IgG- or IgE-binding capacities and the corresponding protein/carbohydrate (P/C) ratios of the antigenic extracts. Low IgG-binding was found in extracts with a low as well as a high P/C ratio (6, Table 3).

Frequently, the changes in antibody-binding capacity of the extracts after addition of YE to the growth medium were not identical for the four fractions of each fungus (mycelial and metabolic antigens of both early and late phase cultures). This can be ascribed to an increase in the growth and metabolism of the fungus after the addition of YE to the culture fluid. Therefore the cultures will be in a different metabolic state when harvested at fixed times of cultivation, as shown in the accompanying paper.

In this study it was demonstrated that the immunochemical properties of the fungal extracts, expressed in precipitation patterns and IgG- and IgE-binding capacities, depend on the time of cultivation. In the light of standardization all four fungi were cultured for 10 and 28 days. It was shown, however, that the growth patterns of *A. fumigatus* and *P. notatum* were rather similar, showing three phases of growth as described before, whereas with *A. alternata* and *C. herbarum* only two phases could be distinguished. Thus, perhaps one fixed time of cultivation could not be used for all fungi. A better approach may be to find the optimal time of cultivation for each fungus in order to obtain fungal extracts with high allergenic potency.

It has often been emphasized that fungal allergenic extracts should contain allergens from different strains to improve the number of IgE-binding components of the extract (13, 14). From this study it is concluded that extracts should contain allergens obtained from mycelial as well

as from culture fluid, isolated from both early and late phase cultures in order to obtain a wide spectrum of allergenic components in the extract.

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Address:

Sicco van der Heide, M.Sc.
Department of Allergology
Clinic for Internal Medicine
State University Hospital
Oostersingel 59
9713 EZ Groningen
The Netherlands

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