Immunological studies on dermatophytes and yeasts: Preparation and standardization of antigens

*Dr. Adnan H.Aubaid AL-Hamadani

summary

glycopeptide fractions isolated from the mycelia (WEAg) and culture filtrate (CFAg) . in addition to , a purified enzymes (PEAg) viz. Keratinase and peroteinase , were prepared as antigens from selected dermatophytes and yeast isolates using alcohol precipitation, ethylene glycol and sephadex G-100 gel filtration methods. The biological standardization of these antigens was also estimated by intradermal inoculation (skin test). Circulating antibody titers was also evaluated using indirect passive haematophytoses and yeast infection .results showed that prepared antigens have .an ability to induce both the cell mediated immune response (delayed type) hypersensitivity test (DTH) and humoral immune response(circulating antibody titers) at diffeent values dependent upon the kind of antigen extracted antigen and fungal isolates used.

الخلاصة

حضرت المستضدات الكلايكوببتيدية المعزولة من الخيوط الفطرية و الراشح الزرعي للفطريات الجلدية و الخمائر المرضية ، بالاضافة الى الزيمات منقاة من نفس الفطريات باستخدام الترسيب بالكحولات و الايثايل كلايكول و الترشيح بالجل .وقيست ايضا " معيارية هذه المستضدات بواسطة التلقيح الجلدي (اختبار الجلد)، فضلا " عن قياس معيار الاجسام المضادة باستخدام اختبار التلازن الدموي السلبي غير المباشر بمصول الارانب المختبرية المحقونة بالمستضدات الفطرية الجلدية والخمائر المرضية . واظهرت النتائج ان المستضدات المحضرة تملك قابلية التحفيز لكلا" من الاستجابة المناعية الخلطية (معيار الاجسام المضادة) و الخلوية (فرط الحساسية المتاخر) وبقيم مختلفة و حسب نوع المستضد المستخلص و نوع الفطر المستخدم

Introduction

Cutaneous mycoses are caused by fungi growing on or within the keratinized parts of the body. Most of dermatophytes and yeast antigens are polysaccharides , proteins , and nucleic acid , but complexes with carbohydrate and protein moieties are common [1] .Earlier , a relible antigen preparation from those fungi are well documented for skin testing as " trichophytin and candidin" [2,3] .such studies have been directed to the reactivity of different antigenic preparations and their value in the hypersensitivity to pathogenic fungal infections.Different procedures have been used hat mainly involve mild chemical extractions such as purification with phenol [4] , hot dilute alkali[5] , and ethylene glycol [6,7] , evidence has been accumulated indicating that the immunogically active major antigenic structure is a specific glycopeptide [8,9]. The aim of this study was to perpare and standardize of different antigens through extraction and purification from mycelia and culture filtrate of selected dermatophyte and yeast isolates.

* Pathology Dept..College of Medicine,AL-Qadisia University,IRAQ

Materials and methods

Patients

Two groups of population have been studies, group A consisting 24 patients aged between 10 – 65 years who had been affected by dermatophtoses and yeast infections; group B consisting of 14 individuals with any type of dermal infection (control group). The mycoses and etiological agents are given in table 1. Clinical specimens and blood collections : A total of twenty four fungal specimens of a clinically active and inflammatory coetaneous lesion were obtained from patient who contracted the in a particular geographic area (southern Iraq) through the period August to October(1999),and attended at the Dermatology and Venereal Diseases Centre , Basrah Hospital. Hairs , nails and skin scrapings were taken and cultured onto Sabouraud 's Dextrose agar (SDA;oxoid)except the skin scraping of the patients with Tinca versicolor was grown on SDA ammended with olive oil (5)% .Incubation was at 27-29 C for 2weeks . Conventional methods of isolation, identification and strain differentiation were used [10, 11, 12], Blood samples were collected at the same time by veinpuncture from all infected and control indivduals and sera were separated by centrifugation , divided into a liquots of 0.5 ml and stored at -20 °C until use.

Fungal isolates cultivation media for antigen preparations

Three species of dermatophytes (Epidermophyton floccosum, Microsporum canis, and Trichophyton mentagophytes var. mentagrophytes) and two species of yeasts (candida a lbicans and Malassezia furfur) were selected for antigens prepartion . For cultivation of dermatophyte and Candida species ,an inoculum of 5 mm disc of fungal stock culture was transferred into 500 ml glucose – peptone broth (glucose 40 g/L.;Bacteriological peptone 10g/L.;PH5.4)in1000 ml Erlenmyer flask without agitation at34°C .Where as M Furfur was grown on media composed per liter :0.06 M ammonium phosphate buffer ,pH5.6;06mg ferroussulfate; 0.13 g magnesium sulfate; 1g potassium nitrate ; 1.3 g sodium chloride ; 3.75 g glycine; 13 g glucose ; 20 ml tween-80, incubated at 29°C.After 14 days of incubation , the contents of dermatophyte flasks were cooled at 4 °C and filtered using Whattman No. 1 filter paper, and by centrifugation (3000 g ,15min) for yeast flasks, then culture filtrates and residual myceiia were stored at 20C until proceed for antigen productions [13,14].

Extraction and purification of antgens from myceiia

Wet myceiium weighing 10 g were ground in a mortor and the macerate was extracted repeatedly using three volumes of water containing 0.5 %phenol at 70°C for 30 min with mechanical stirring , then concentrated with 50 ml of aceton then extracted with 500 ml ethylene glycol[15] .After 24 h , the thylene glycol was separated from the myceiial material by filtration.The extract was dialyzed against a running tap water and after centrifugation, the solution containing the extracted material was lyophilized (lyophilizer,Virtis Co. ltd.,USA). The yield was coded as a whole cell extract antingen (WEAG).

Extraction and purification of antigen from culture filtrate

Two liters of culture filtate were dialysed against running tap water for one week in a Viskin cellophane tube to remove the metabolites and the medium's micromdecude ingredients [16] The dialysate was concentrated to 100 ml under lyophilization. The glycoproteins were isolated from the filtrates by alcohol precipitation(75%thanol).The yield was coded as culture filtrate antigen(CFAg).

Isolation and partial purification of enzymes as antigens

Acolumn of sephadex G-100 was employed after being buffered and washed with a suitable buffer for partial purification of keratinase and proteinase from the culture filtrate of Trichophyton mentagrophytes var. *erinacei* [17,18]. The pooled elute dialysed and lyophilized . The yield was coded as a purified enzyme antigens(PEAg).

Estimation of antigden titer

Antigen solution : Each antigen was dissolved separately in sterile water to give a 0.1% solution and used as stock antigen solution.

Experimental infection of rabbits

Isolates of the selected fungi which have been used for extraction of the antigens, were employed for experiment infection of rabbits (local strain)by intradermal inoculation. After infection takes place , hair and skin scraping were obtaind and cultivated in sabourauds Dextrose broth for2weeks at 27°C , then mycelium harvested and macerated with the physiological salution . The macerate was inoculated intraderated with the physiological saline solution. The macerate was inoculated intradermally into the back of rabbit (weighed 1.5-2.5 kg) to Induce coetaneous mycoses[16] The assays were performed with two lots (infected and control)of three rabbits for each isolates.

Preparation of antisera

Rabbits were infected as mentioned above, and an antigen solution (2ml of 0.1% solution) was mixed with equal volumes of Fruends complete adjuvant (Difco Lab.) to give an emulsion which was used for intradermal inoculation of rabbits at five sites corresponding to the major lymph nodes . The rabbits were reinoculated after one month at two sites in the shouldders using 2 ml of 0.05% antigen solution , but mixed with Fruend's incomplete adjuvant.Blood obtained from the marginal ear vein of the rabbits after one further booster inoculation (after the third weeks) was allowed to clot and the antisera collted and stored at 20°C in 5 ml aliquots after inactivation at 56°C for 30 min [15]

Intradermal reaction (skin test)

Rabbits were infected as mentioned above, and an antigen solution (0.1ml of 0.1% solution)was injected intradermally into the back of infected rabbits at

a distance of 5 cm from the margin of the infected area . Apositive intradermal reaction (appearance of redness)and the diameter of redness was measured at 24 to 48 h after the injection of the antigen [16].

Estimation of antibodies titer

Two hundred and fifty ml of phosphate buffered saline (PBS; PH7.2) wasplaced in 20 well of a perspex hemagglution tray, except for the first left well in which another 200 ml were added. To this well 50 ml of serum sample of rabbit was added, mixed well and 250 ml was transferred to the next well.this wa repeated reaching the last well where 250ml were discarded. 250 ml of sheep RBCs coated with Ag wher added to each sample well .The trays were agitated carefully, covered with parafilm and read after 18 h incubation at 4°C The agglutination titer is the highest dilution of serum which brings about aggreation of the particles [19]. All data were statistically analysed using analysis of variance table.

Results

Two – types of glycopeptide fractions which extracted from the mycelia and culture filtrate of selected dermatophytes and pathogenic yeast isolates, and two purified enzymes (keratinase and proteinase) which parially purified from Trichophyton mentagrophytes var. erinacei,were prepared as antigens. The infected rabbits with experimental dermatophytoses and yeast infections by different isolates were positive, with exception of Malassezia furfur which was negative. All the prepared antigens were subjected to the measurement of their antigenic titer by the intradermal reaction (skin test) the average diameters of the redness area caused by the injection of each antigen are given in table (2).All the tested antigens gave more or less positive reaction , except for M. furfur was negative . Generally , purified enzymes (PEAg) showed more intesive reaction (9-17mm)followed by CFAg (8-15mm)and lowest was WEAg (6-9mm).Significance differenced ($p<0.05$) between these reactions were observed .Among the fungal isolates , Trichophyton mentagrophytes var. mentagrophytes antigens elicited remarkably strong reaction (9-17mm)and weakness reaction (7-9mm)for Candida albicans antigens (Table2).The differences were statistically significant ($p<0.05$). Hemagglutination reaction was performed on each of the prepared antigens to evaluate the agglutinations antibody titers of rabbit sera and to compare the antigenicity in the intradermal reaction with that of agglutintion reaction (Table 3). Signifcace antibody titers($p<0.05$)were observed against WEAg of Microsporum canis (163840)and lowest (5120)against PEAg of *C. albicans*. The results demonstrated that the agglutination reaction and intadermal reaction were strong and significant ($p<0.05$).Comparatively,WEAg showeda weak intradermal reaction (+)but it gave a strong agglutination reaction (+++)(Table4).

Discussion

Generally ,the immune response to fungi are cell- mediated one, it seems to be more relevant to measure the specifity and sensitvity of antigens production .However, among the purified fractions of dermatophytin (complex of metabolic products) activity, the most frequently encountered are the polysaccharide substances which linked with small quantities of the peptide components [20]. The developing a pure antigen with a minimum of non-specific properties still limited [20].The present study revealed that the dermatophytin activity does not related to a single antigen with a definite chemical constiutes, but is a rather a function of different types of substances including polysaccharides, polypeptides and nucleic acid with varing intensities. This finding supports the hypothesis of multiplicity of dermatophytins as presented earlier [22]. The WEAg fractions which gave a mild intradermal reaction may reflect that the simple peptides containing fractions which are an important contribution of the antigenic substances in the manifestation of the intradermal reaction [23].While the PEAg fractions which produced a strong intradermal reaction can be attributed to the presence of a complex of polypeptides as stated earlier[16].Dermatophytin was considered to be a complex metabolic product, something like an "endotoxin " of the fungus which would be liberated into the culture medium only if destruction of the fungal took place . This may explain that the dermatophytins present in the culture medium seem to have a similar composition as those obtained from the fugal mycelium in this study such as amino acid as well as sugars [16]. The reaction of rabbit sera with the prepared antigens appeared to be related to the type of antigen extracted and the fungus .Thus , highly antiboy titers against WEAg fraction (particulate antigen) when compared with PEAg (soluble antigen)using hemagglutination test may be related to the physical nature of the antigen .However ,the larger size of the antigen,the fewer molecules of antibody are required to bring about their visible aggregation .It is also stated that crude antigen preparation showed higher antbody titer than the purified antigens[1]. The fact of non-parallelism observed between intradermal and agglutination reaction among different antigens may explain that even the fractions giving rise to a weak intradermal can sometimes be an effective antigen for the agglutination reaction .Such results supports the earlier study [24]which reported that the Microporum canis antigens with the highest inflammatory potential was the most able to produce a humoral response and Trichopyton mentagrophytes was the most able to produce a dermal response.

Table(1) List of mycoses ,etiological agents and number of cases studied.

Mycoses	Etiological	Source	No.of Cases
Tinea pedis	Epidermophyton floccosum (Harz) Lancrosporum & Mliochev	Skin	4
Tinea capitis	Microsporum canis(Bodin)Bodin	Hair	5
Tinea corporis	Trichophyton rubrum (Castell.)Sab.	Skin	4
Tinea cruris	T.mentagrophytes var mentagrophytes	Skin	5
TineaVersicolor	Malassezia furfur(Robin)Baillon	Skin	3
Cutaneous Candidiasis	Candida albicans(Robin)Berk	Nail	3
Total			24
Control group			14

Table (2) intradermal reaction to the prepared antigens extracted from dermatophyte and yeast isolates.

Etiologial agent	Intradermal reaction		
	Diameter of dermal response (mm)to antigens		
	PEAg (X ± SD)	CFAg (x±sd)	Weag (x±sd)
E. floccosum	15±2.0	10±1.4	6±0.2
M. canis	13±1.5	10±1.4	7±0.2
T. mentagrophytes Var.mentagrophytes	17±2.2	15±1.5	9±0.3
C.albicans	9±0.2	8±0.3	7±0.2
M.furfur	0.0	0.0	0.0

Table(3) Indirect passive hemagglutination reaction of the rabbit sera to the prepared Ags.

Etiological agent	Antibody titers		
	PEAg	CFAg	WEAg
E. floccosum	10240	40960	40960
M. canis	40960	81920	163840
T. mentagrophytes Var. mentagrophytes	20480	40960	81920
C.albicans	5120	10240	20920
M . furfur	0.0	0.0	0.0

Table(4)Comparative results of intradermal and agglutintion reactions to the prepared antigens.

Antigens	Intradermal reaction	Agglutionation reaction
WEAg	+	+++
CFAg	++	++
PEAg	+++	+

(−)Weak reaction
(−−)Moderate reaction
(−−−)Strong reaction

References

1. Mackenzie.D.W.R.(1983)Serodiagnosis.In:Fungi pathogenic for humans and animals.partB:pathogenicity and Detection , D.H.Howard (ed).vol.3.,Marcel Dekker, USA.pp.121-218.
2-Aas. K. (1980) purification and characterization of allergen preparation and standardization of materoals and methods in allergy .Allergy 35,165-167.
3-weeke.B.(1980)Standardiztion of allergen preparation .Allergy 35,172-176
4-Nozawa,Y.;Kitajima,Y.and Ito .Y.(1973)Chemical and ultrastructural studies of isolated cell walls of Epidermophyton floccosum .presence of chitin inferred from X-ray diffraction analysis and electron microscopy .Acta Biochem .Biophys.Acad.Sci Hung.307.92-103.
5.Bishop.C.T.;Blank ,F.and Hrranisavljevic –Jakovljevic,M.(1952)The water-soluble oplysecharides of dermatophytes I.A galactomannanfrom Trichophyton granuolosum .Can .J.Chem .40,18161-1825.
6-How.M.j.:Withnal ,M.T.and Cruickshank ,C.N.D(1972)allergeenic glucans from dermatophytes .part I.Isolation, purification and biological properties .Carbohydr .Res.25,314-353.
7-Ottaviano ,P.J.;Jones ,H.E.;Jaeger ,J.;King ,R.Dand Bibel,D.(1974)fromTrichophyton mentagrophytes grown in a complex medium and a defined medium . Appi.Environ.Microbio 1.28,271-275.
8-Grappel.S.F.;Bishop,C.T.and Blank.F.(1974)Immunology of dermatophytoses. Bacteriol .Rev.38,222-250.
9-Weitzman,I.and Summerbell,R.C.(1995)The dermatophytes.Clinic Microbiol.Rev.8,240-59.
10-Ajello,L.(1968)A taxonomic review of dermatophtes and related species Sabouraudia 6.147-159.
11-Ellis,D.H.(1995)clinical Mycology:The human opportunistic mycoses. Gillingham printers pty Ltd .Australia.p.166.
12- Hoog de.G.C.and Guarro,J.(1995)Atlas of clincal fungi. Centraalbureau voor Schimmekultures,Unversitate Rovira Virgill,Netherland,Espain.pp.679.

13- Sanyal,A.K.; Dsa,S.K.and Banerjee,A.B.(1985) purification and purification and partial characterization of an exocellular fromTrichophyton rubrum Saburaudia 23,165-178.

14-Ghannoum,M.and Abu Elteen,K.(1986)Correlative relationship between proteinase proteinase production ; aldherence,and patholgenic of various stains of Candida albicans . Journal of medical and veterinary mycology 24,407-413.

15-Hann de . p.: Van der .E.M.H. and Boorsma . D.M.(1986)Diversity of antigenic extract from the dermatophte Trichophyton rubrum Mykosen 30,427-433.

16-Ito,Y.(1965) on the immuological active substance of the dermatophytes,J. invest. Dermatol.24,275-295.

17-Aubaid ,A.H.and Muhsin ,T.M.(1997) Partial purification and kinetic studies of exocellular proteinase of Trichophyton mentagrophytes var. erinacei .mycoses 458 10-17.

18-Muhsin ,T.M. and Aubaid ,A.H.(1997) Partial purification and som biochemical characteristics of exocellular keratinase form trichophyton mentagrophtes var . erinacei. Mycoses31:21-27.

19-Reyes ,A.C. and Friedman ,L.(1966) Concerning the specificity of dermatophytes – reacting antibody in human and experimental animal sera.J.Invest.dermatol.47,27-34.

20- Kaaman, T.(1985) dermatophte antigens and cell-mediated imunity in dermatophytosis .in : current topics in medical mycology ,M.R.Mcginnis (ed.) vol.1. springer verlag. new York ,USA.PP.117-154.

21-Kaaman , T.(1988) hand,foot and nail disease : a common manifestation of chronic dermatophytosis .mycoses 31,613-616.

22-Kull,E.;Brun, R. and Jadassohn ,W.(1951)A proposed trichophytines seches .int.arch.allerg.2,252.

23-Merkel,M.(1957) The chemical and antigenic structure of Trichophyton gypseum .Bull .Acad .pol.sci.(Biol.)5,341.

24-Roig,A.M.and Rodriguez .M.T.(1987).The immune response in childhood dermatophytoses . Mykosen 30, 574-580.