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THE ROLE OF POWDER MILK AS ACAUSATIVE AGENT OF TYPE ONE HYPERSENSITIVITY AND PREPARATION OF ALLERGY VACCINE.

Othman R.M.
Department of microbiology,College of Veterinary Medicine,University of Basrah,Basrah,Iraq
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ABSTRACT

The protein extract from powder milk were prepared by extraction, followed by purification and fractionation using gel filtraction. One peak was obtained from powder milk with molecular weight of 22KDa.

ELISA and skin test were performed on 195 patients tested with powder milk.The rate of positive results to skin test and ELISA was 60.51%.

There were significant differences $P<0.05$ among age groups regarding the number of patients who had positive skin test and ELISA results ,the mean value of flare diameter and OD-values, in addition, a significant differences $P<0.05$ occurred among males and females examined with powder milk .

INTRODUCTION

Allergy to cow's milk protein [(CMPS)] occurs principally in the first half year of life coinciding with its introduction into the infant's diet IgE-mediated reactions are the best known and characterized of all food allergy reactions[7] Diagnosis of immediate hyper-sensitivityto cow milk protein is based on the clinical background and on the demonstration of specific IgE antibody for CMP, however the challeng test will either confirm or reject the presence of clinical symptoms[4]. Once the presence of clinical reactivity is reified, the only treatment is a diet excluding this protein and the administration of substitute formula[8] Thus, it is very important to use the challenge test to verify the diagnosis, however it must be remembered that these tests are uncomfortable for the patient ,take time, and are not free from undesirable effects.Therefore, it is necessary to find methods that will make it possible to avoid using those challenges that have a high probabitity of having positive responses. The skin test is the first choice to investigate immediate hypersenstivity reaction because it has great sensitivity[20,24]

The quantification of the specfic IgE antibodies in serum with ELISA is reported to have improved sensitivity [11]
Some studies have found an association between higher level and of specific IgE and clinical reactivity [20,23]

The purpose of this study was to determine the total and specific IgE levels in the diagnosis of immediate hypersensitivity to commerciall full cream milk powder.

MATERIALS AND METHODS

Patients selected for intradermal skin testing comprised (195) individuals 79 males and 116 femals of eligible cases attending the center of asthma and allergic diseases in Basrah ,aged between (10-60) years. All patients have a symptom related to upper or lower respiratory tract disorders or conjunctival diseases or urticaria.

Preparation of milk powder antigen:-

Milk powder (vinamix company, veitnam)imported by the ministry of Trade, state co. for food stuff trading. Milk powder extract was prepared as discribed by (Garcia-Ara *etal*[11]) The milk powder was mixed with phosphate buffer saline (PBs,0.15M,PH 7.2) 5/100 w/v then calrified by refrigerated centrifuge at 10.000 rpm for 1 hour at 4C° .The

supernettant solution was sterilized by milipore filter (0-45 µm)and stored at 4C°. The protein content of the milk powder extract was estimated according to whitaker and Grannm [28]method.

The purification and fractionation of milk powder extract on G-75 sephadex:-

Gel-filtration –liquid chromatography was used to fractionate and purify the extract into molecules of different molecular weight according to the method of leslie and Frank[17].

Determination of the sterility and safety of milk powder extract :-

The sterility and safety of the extract was determined according to method of Maeckie and Maccartney[18] The sterility was determined by inoculation of the extract into duplicate plates of nutrient and blood agar. Then these plates was incubated aerobically and anaerobically at 37C°. The safety of the extract was estimated by inoculation of 6 rabbit (1-1.25 kg) with (2.8-3ml/rabbit) intramuscularly, other 6 rabbit were used as control group .Inoculated plates and rabbit were observed dialy for 7 days after inoculation to determine the culture sterility and to obseve any nervous symptomes or behavior exchange of rabbit .

***In vivo* tests**

Intradermal skin tests (ID) was performed according to astandard procedure with milk powder extract (5%wt/vol)(Drebery *etal*[9]. Histamine diydochlorid diluted 1:100 was used as a positive control and glycerosaline was used as anegative control .Reactions were read in15 minutes .A net wheal diameter 3 mm larger than that produced by anegative control was considered positive .

***In vitro* test**

Specific IgE antibodies to milk powder were measured by commercially available enzyme–linked immunosorbent assay (Biomaghreb Kit. Tonisia)

Specific ELISA technique

Specfic IgE was determined according to the method of Biomaghreh Kit .Briefly, the reference discD allergen (Dermat pteron) were added to wells of microtiter plate started from3rd well of first vertical row to 8th well of second vertical row followed by the addition of reference serum calibrator (A-H) in which IgE concentration was (52,50,17.50,0.70 and 35 ML) to the reference D disc .The 50 ml of powder milk extract (1,300) was added to the rest of wells and 50 ml of patients sera (1/20) was added to these wells-plates were then covered with plastic film ,homogenized by shaking at 300 rpm and incubated at 37c° for 90 minutes followed by washing with PBS-tween 20(0.05%). After washing ,(100ML) goat anti-human IgE alkaline phosphate conjugates (1/100) was added to each well.The plates were then covered with plastic film and incubated at 37c° for 90 minutes. After that, the plates were washed and freshly prepared para-nitro phenyle-phosphate solution (100ML) was added to each well.Then the plates was incubated at roon temerature for 30 minute in the dark and (100ML) of the stopping solution (N NaoH) was added to each well. The absorbance of each well was read at(450 nm) using microplate reader (Dynatch, microplate reader, model SMR 600,U.S.A).

Statistic

For the determination of statistical significant of ELISA and skin test results.Qi-sequre (x^2) test was used.

RESULTS

The protein extracts from powder milk were purified and fractionated by gel filtration using sephadex (G-75), One major peak was observed fig(1) .The molecular weight of the purified protein and concentration in relation to the original protein content were reported in table (I) .The molecular weight of the purified antigen was estimated by measuring the elution volume of some standard proteins fig(2),the kav values were calculated and plotted versus the logarithm of their molecular weight .On the other hand

the allergenic activity and clinical relevance of the powder milk protein extracts were assayed in 195 patients using intradermal skin testing reported in table (2) .In this table, the highest rate of positive results was observed in males examined with powder milk (65.82%) in compare to femals(56.89%).

Table 3 shows the rate of skin test result in 195 patients examined with allergens of powder milk , the figure of this table have shown that the highest rate of positive response were observed in the first age group in both sexes. The rate of distribution was decreased gradually in other age groups in both sexes .Table 4 have shown that the skin test reactivity in the different age groups is increased progressively with age and decreased gradually beyond age of 25 years old .The skin test reactivity was estimated by the mean value of flare diameter .In addition, table 2 shows that there is no difference between the positive results of skin test and ELISA.On the other hand, there were asignificant differences $P<0.05$ among males and females examined with powder milk according the number of patients who had positive ELISA results. Table 4 shows the means ±SD of the OD values of positive serum samples which were tested with powder milk allergen using IgE-based ELISA.In this table, we can observe that the patients who have positive skin test responses also showe high and moderate OD values and there were no difference between age groups or males and females concerning their OD values .

Table (1) The protein content and the molecular weight of crude protein extracts of powder milk .

Source material	Protein content crude extract mg /ml	Protein concentration% MW(KD)
Powder milk	2.9	0.219/22

Table (2) Rate of skin test and ELISA positive results in 195 patients examined with powder milk

Protein extract	+ve response No.(%)		Total
	Males	Females	
Powder milk	52/79(65.82)	66/116(56.89)	118/195(60.51)

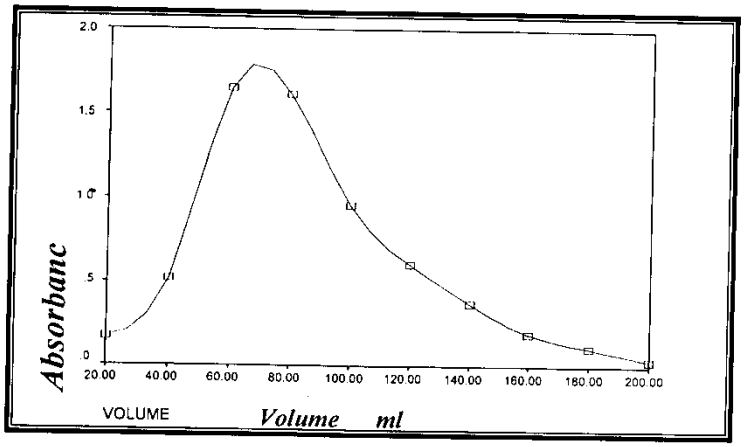

Fig. 2 Elution profile of powder milk

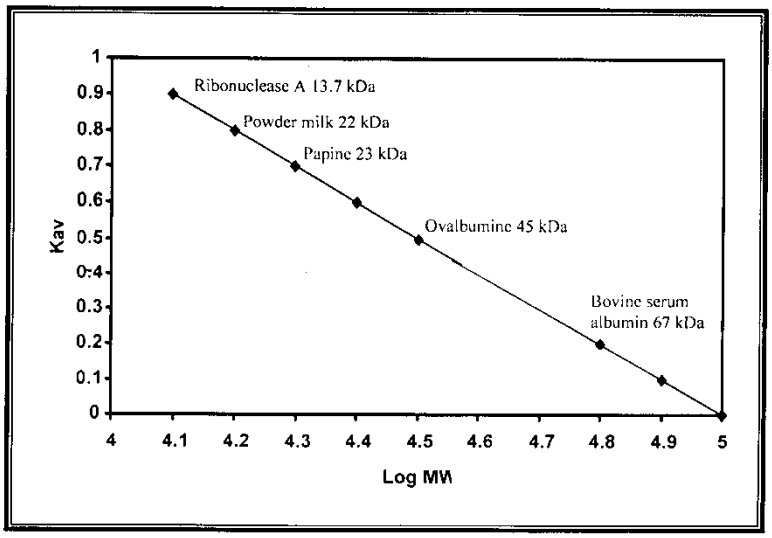

Fig.3 The calibration curve of protein extract using some standard protein for estimation of protein molecular weight

Table (3) Rate of skin test and ELISA results in 195 patients tested with allergen of powder milk

Age groups years	Male			Female			Total
	Exam. No	Skin test +ve No.%	ELISA+ve No.%	Exam. No.	Skin test +ve No.%	ELISA +ve No. %	
>10-20	24	21(87.50)	21(87.50)	35	27(77-14)	27(77-14)	59
>20-30	20	14(70.00)	14(70.00)	32	19(59.37)	19(59.37)	52
>30-40	18	11(61.11)	11(61.11)	29	14(48.27)	14(48.27)	47
>40-50	17	6(35.29)	6(35.29)	20	6(30.00	6(30.00)	37
	79	52	52	116	66	66	195

Table (4) skin test reactivity and ELISA results of 118 patients with positive response to powder milk.

Age group years	Males		Females	
	Dm mean±SD	OD IgE p.m.	Dm mean ±SD	OD IgE p.m
>10-20	25.9±3.5	0.91±0.43	23.7±4.7	0.85±0.48
>20-30	21.3±5.5	0.72±0.37	20.0±7.3	0.67±0.43
>30-40	18.8±6.7	0.50±0.53	16.1±6.3	0.47±0.39
>40-50	13.0±6.9	0.34±0.29	11.8±5.6	0.29±0.2

DM=Mean diameter value of flare (mm.)

PM=powder milk
OD=optical density

Table (5) The chequar-board ELISA titration

Source material	Final dilution				Back ground values	Negative cut off
	Antigen		Antiboly	Conjugate	IgE based ELISA-OD	IgE based ELISA-OD
	Crude extract	Purified extract	IgE	IgE-based ELISA		
Powder milk	1/100	1/300	1/20	1/100	<0.073	0.036

OD= optical density values.

DISCUSION

Protein purification :-

In the present study, One major peak were demonstrated by gel filtration analysis of protein extracts of powder milk protein ,which represents a major allergen ,the molecular weight of the eluted protein was 22KDa , this result was in line with the results of Week and lowenstein[27] ,Aukrust and Aas[1],Groot *etal*[12],who reported that the major allergen is an antigen to which more than 50% of allergic patients have IgE antibodies in their sera. In addition ,half of them should have high levels of the antibody, On the other hand the molecular weight of eluted protein was in line with the finding of Drebory *etal*(10), March and Norman[19], who reported that the allergen molecule usually has a molecular weight of 5.000-70.000 Daltons.

Skin test reactivity

The rate of ELISA and skin test positive results in patients examined with powder milk was high in compare to the finding of Bock and Atkins[3].The explanation of this discrepancy is based on differences in geographic area, climates and genetic factors Breiteneder and sheiver[6].Other factors that should be taken into consideration is the particular skin –test technique used the site used for testing ,age ,sex and race of the patient Pastorello[21].Also there was a significant difference $p<0.05$ among age groups of patients examined with powder milk regarding the rate of the flare diameter and the rate of positive skin test results and these results were in agreement with the results of Bock[2]. Sampson and Scanlon[25]. Hattevig *etal*[14] ;kanny *etal*[16] ,who reported that the skin test reactivity acquired progressively during childhood ,between 15-25 years and declining gradually. There was also a significant difference $p<0.05$ regarding the rate of positive responses among females and males examined with powder milk antigen and this finding was in agreement with other studies kanny etal [16] , who found that the rate of positivity in patients up to 15 years of age is more frequent in males.

ELISA-technique

The rate of ELISA positive results is resembling that of skin test and this finding was in line with other studies (Varjonen *etal*[26].; Haaltela and Jeakonmaki[13],who reported same rate of positivity in ELISA and skin testing.

In conclusions,the most important allergens of powder milk extract was eluted in one major peak which represent the major allergen,according to skin test and ELISA resultes ,the protein extract of powder milk have allergenic activity.

دور الحليب المجفف كمسبب للنوع الاول من فرط الحساسية وتحضير لقاح الارجية

رشا منذر عثمان

فرع الاحياء المجهرية،كلية الطب البيطري،جامعة البصرة،البصرة،العراق

الخلاصة

تم تحضير واستخلاص المحاليل البروتينية من الحليب المجفف وتنقيتها وتجزئتها بواسطة الترشيح الهلامي اذ تم الحصول على قمة واحدة من الحليب المجفف وبوزن جزيئي ٢٢ دالتون.

اجري فحص الجلد والـــELISA على ١٩٥ مريضا تم اختبار حساسيتهم للحليب المجفف وكانت نسبة المرضى الذين اظهروا النتائج الموجبة هي ٦٠,٥١% وقد وجد بأن هناك فرق احصائي معنوي P>0.05 بين الفئات العمرية فيما يخص عدد الاشخاص الذي اظهروا نتائج موجبة في اختباري فحص الجلد والـــELISA ومتوسط قطر الاحمرار الجلدي ومتوسط قيم الكثافة البصرية.كذلك وجد فرق احصائي معنوي P>0.05 بين الذكور والاناث المتحسسين للحليب المجفف

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