

***In-vitro* responses of leucocytes to food proteins in allergic and normal children: lymphocyte stimulation and histamine release**

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Summary

In vitro quantitative methods for determination of antigenic release of histamine from leucocytes and stimulation of small lymphocytes to transformation into blast forms were applied to a study of immunologic responses to food proteins in allergic and non-allergic children. The allergic group was subdivided according to positive or negative reactions to intradermal injection of the food proteins.

Significant lymphocyte stimulation by proteins from foods universal in the diets, like cow's milk and eggs, occurred as frequently in non-allergic as allergic children, with or without positive skin tests to the food proteins. Furthermore, the average stimulation among the groups was not significantly different.

A soybean protein gave significantly greater stimulation of lymphocytes from allergic children, in whose diets soybean formulae are frequently substituted for cow's milk.

Antigenic release of histamine from leucocytes occurs only with leucocytes from skin-test positive persons, and in only about 25-50% of these in the case of food proteins.

Neither the procedure for lymphocyte stimulation nor for histamine release from leucocytes, as carried out in this study, will serve to determine unequivocally whether a particular food protein is causing symptoms in question.

Introduction

The purpose of this study was to find out whether *in vitro* reactions of cells from persons hypersensitive to food proteins may identify the role of foods in allergic disorders, and to add to the knowledge of immunologic responses in food hypersensitivity.

Antigenic stimulation of lymphocytes and histamine release from leucocytes are cellular reactions involving different immune mechanisms, and this should favour detection of hypersensitivity to food proteins. Quantitative methods for *in vitro* measurement of these reactions were available.

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Food proteins selected for study were from foods commonly consumed from early infancy, and some were obtainable as purified proteins.

Subjects

The studies were conducted with leucocytes from allergic and non-allergic infants and children 1 month to 16 years of age (the majority between 6 and 12 years), receiving care at Bellevue Hospital, New York, N.Y. Clinical histories and physical findings regarding allergy were obtained by the authors.

Not symptomatic, not atopic is the designation for subjects free of allergic manifestations by personal history and physical findings, and who exhibited neither immediate weal nor delayed reaction to intradermal injection of about 0.01 ml of extracts of common allergens. Allergen extracts injected were at concentrations up to 1000 PNU/ml* (10 µg protein nitrogen), and included cow's milk proteins, ovalbumin, wheat, ragweed, alternaria and house dust.

Symptomatic, atopic is the designation for allergic subjects with definite allergic symptoms (allergic rhinitis, asthma, eczema) and immediate type weal reactions (diameter 2 mm or more greater than bleb from control injection) to intradermal injection of extracts of one or more of the common allergens already mentioned; usually multiple allergens gave positive reactions.

Diets of the subjects were with few exceptions unrestricted at the time leucocytes were obtained for study.

Methods

Lymphocyte stimulation

Lymphocyte stimulation was measured as previously described (May, Lyman & Alberto, 1970a). In this procedure leucocytes were separated by gravity from heparinized blood mixed with 3% solution of Dextran (mol. wt. 280,000) in a 5:1 ratio. The leucocyte-plasma layer obtained from 10 ml blood was diluted to 49 ml with culture medium to provide 2 ml of cell suspension in twenty-four tubes; there were $5-10 \times 10^5$ lymphocytes/ml. We found that lowest control values for lymphocyte transformation occurred when autologous plasma was the source of protein, and that Dextran as used did not affect the antigenic stimulation of lymphocytes. Cultures were carried out in duplicate and twenty-four tubes permitted testing of four 10-fold concentrations of two food proteins and three of a third protein along with the control tubes without antigen. Maximum stimulation almost always occurred with concentrations between 2 and 2000 µg food protein/ml. Results are in terms of counts per minute (cpm) from tritium-labelled thymidine incorporated into lymphocytes during transformation; the thymidine being introduced into cultures on fifth day of incubation, and the cells harvested 24 hr later. Degree of lymphocyte stimulation is expressed by the ratio:

$$\frac{\text{Mean cpm of culture exposed to antigen}}{\text{Mean cpm of unstimulated control culture}}$$

This ratio indicates the number of times greater is the transformation of lymphocytes stimulated by antigen than the spontaneous transformation in a control culture to which antigen was not added.

* 1 Protein Nitrogen Unit (PNU) = 0.01 µg protein nitrogen (PN).

Antigenic release of histamine

A previously described procedure for measurement of antigenic release of histamine from leucocytes was employed (May *et al.*, 1970b). From 10 ml blood a sufficient quantity of leucocytes (basophils) was obtained to determine histamine release with eight concentrations of antigen. The range of concentrations used was 0.2–2000 μg protein/ml, which experience had shown would detect maximal histamine release from leucocytes of subjects with the variations in degree of hypersensitivity encountered. Histamine release is expressed as per cent of total histamine in an aliquot of the leucocyte suspension.

Materials

Allergen extracts for skin testing

Stock extracts purchased from Center Laboratories, Port Washington, New York, were used. These were phosphate-buffered saline extracts containing 0.4% phenol and were of 20,000 or 40,000 PNU/ml concentration. Fresh dilutions of 100 and 1000 PNU/ml were prepared every 2 weeks with phosphate-buffered saline containing 0.4% phenol which was obtained from the same company. All extracts were stored at 5°C. Skin tests were performed by intradermal injection of these dilutions.

Antigens for lymphocyte stimulation and histamine release

Cow's milk proteins. Mixture prepared from purified proteins from Pentex Division of Miles Laboratories: Crystallized bovine serum albumin (BV0162); casein (PP0262); alpha-lactalbumin (PP1262); and 3 $\times$ crystallized beta-lactoglobulin (PP0622). 110 mg of each was dissolved in 20 ml tris-buffer solution prepared as described below, to give a stock solution of 2.2×10^4 μg of mixed milk proteins/ml.

Ovalbumin (chicken), 5 $\times$ crystallized, from Pentex (PP0662). Stock solution prepared to contain 2.2×10^4 μg /ml.

Wheat extract. Extract of ground whole wheat, lyophilized (provided by courtesy of Center Laboratories). Reconstituted in tris buffer to give stock solution of 1000 μg protein nitrogen/ml.

Soybean extract. Commercially-produced dried protein extracted from soybeans (Edi-Pro A), commonly used in preparation of soybean formulae employed as substitutes for cow's milk in infant feeding. Stock solution prepared to contain 2.2×10^4 μg /ml.

Tris-buffered saline (concentrated). 2.95 g Trizma 7.7 (Sigma Chemical Co.), crystals of tris (hydroxymethyl) amino-methane pre-set to give solution of pH 7.7 at 25°C. 7.59 g sodium chloride. To 100 ml with distilled water. Diluted 1:10 with distilled water, autoclaved, and 100 Units penicillin and 100 μg streptomycin/ml added aseptically before use.

Preparation of solutions of antigens. Solutions and dilutions of antigens were prepared with the sterile tris-buffered saline, which contains penicillin and streptomycin in same concentrations as medium used for culturing lymphocytes (May *et al.*, 1970a). Detectable sensitivity to these antibiotics is rare in children, and controls with the buffer alone were performed in each experiment for histamine release and lymphocyte stimulation; no effect of the antibiotics was ever found. Phenol was not used in antigen solutions because it interfered with both leucocyte histamine release and lymphocyte culture. Stock solutions of antigens were prepared freshly every 2 months and were

sterilized by filtration (Swinnex membrane, 0.45 μ pores, Millipore Filter Corporation); fresh dilutions were prepared aseptically every 2 weeks. Stock solutions and dilutions were stored at 5°C.

Results

Antigenic stimulation of lymphocytes

Significant stimulation. In cultures of lymphocytes some 'spontaneous' transformation to blast forms is always detected in controls or without the addition of antigen in all subjects, as judged by incorporation of tritium-labelled thymidine. To be meaningful the difference in incorporation of thymidine between the stimulated and unstimulated or control cultures must be significantly greater than the variations in duplicate control cultures. In 225 cultures by our procedure values for

$$\frac{\text{Difference in cpm of duplicate controls}}{\text{Mean of cpm of duplicate controls}}$$

averaged 0.125, S.D. 0.111. As $2 \times \text{S.D.} = 0.22$, we considered significant stimulation by antigen to have occurred if a value greater than 1.50 was obtained for the ratio

$$\frac{\text{Mean cpm of duplicate cultures with antigen}}{\text{Mean cpm of duplicate control cultures}}$$

We found with the procedure employed that replicate cultures of lymphocytes (on the same day or weeks apart) from individuals whose lymphocytes were not stimulated by an antigen always yielded values for this ratio less than 1.50. With our procedure, the values for this ratio with phytohaemagglutinin stimulation are in the range of 75–300, and for stimulation by purified tuberculin protein derivative are in the range of 10–100 in tuberculin-positive persons.

Food proteins. The findings from addition of food proteins to cultures of lymphocytes from symptomatic, atopic (or allergic) children and not symptomatic, not atopic (or non-allergic) children are presented in Tables 1–4. The symptomatic, atopic group is divided into those who gave a positive weal reaction to intradermal injection of the food protein tested in cultures of their lymphocytes and those who had negative skin tests for the food protein added to the lymphocyte cultures. An atopic subject may have had a positive skin test to one or more of the food proteins, and always had positive skin tests to one or more inhalant allergens. Symptoms in the symptomatic, atopic group were not necessarily produced by the food protein which gave a positive skin test.

Inspection of the data in Tables 1–4 will reveal that the range of values for maximum lymphocyte stimulation was wide in all groups and the highest value encountered with each food protein was generally found in the symptomatic, atopic group. In all the groups a large percentage of children showed a significant increase in stimulation of their lymphocytes with each of the food proteins.

Furthermore, the mean values for increase in lymphocyte stimulation in groups A, B and C in the Tables were not found significantly different by statistical analysis in the case of milk proteins and ovalbumin (Tables 1 and 2).

These findings indicate that stimulation of lymphocytes from the non-allergic children by these food proteins was comparable to stimulation of lymphocytes from

Table 1. Lymphocyte stimulation by cow's milk proteins

	Skin test with cow's milk	
SYMPTOMATIC, ATOPIC*	Positive (Group A)	Negative (Group B)
No. of children	28	16
No. of assays	40	19
Maximum stimulation by antigen:†		
Significant increase	25/28 (89%)	16/16 (100%)
Range	0.83–12.95	1.50–5.69
Mean	3.86	2.62
S.D.‡	2.81	1.42
Mean A v. B, $P > 0.05$		
NOT SYMPTOMATIC, NOT ATOPIC*		(Group C)
No. of children		16
No. of assays		19
Maximum stimulation by antigen:		
Significant increase		14/16 (87%)
Range		0.99–9.40
Mean		3.44
S.D.		2.27
Mean A v. C, $P > 0.05$		
Mean B v. C, $P > 0.05$		

In Tables 1–4:

* Defined in section on subjects.

† Ratio of cpm of stimulated and control cultures; significant increase is 1.50 times control as explained in text.

‡ Standard Deviation.

Table 2. Lymphocyte stimulation by ovalbumin

	Skin test with ovalbumin	
SYMPTOMATIC, ATOPIC	Positive (Group A)	Negative (Group B)
No. of children	17	23
No. of assays	25	33
Maximum stimulation by antigen:		
Significant increase	13/16 (76%)	17/23 (73%)
Range	0.86–3.82	0.86–7.72
Mean	2.05	1.86
S.D.	0.80	1.03
Mean A v. B, $P > 0.05$		
NOT SYMPTOMATIC, NOT ATOPIC		(Group C)
No. of children		14
No. of assays		16
Maximum stimulation by antigen:		
Significant increase		9/14 (64%)
Range		0.88–3.70
Mean		1.97
S.D.		0.87
Mean A v. C, $P > 0.05$		
Mean B v. C, $P > 0.05$		

Table 3. Lymphocyte stimulation by wheat

	Skin test with wheat	
	Positive (Group A)	Negative (Group B)
SYMPTOMATIC, ATOPIC		
No. of children	12	22
No. of assays	19	29
Maximum stimulation by antigen:		
Significant increase	12/12 (100%)	17/22 (77%)
Range	1.77-16.98	0.92-13.46
Mean	5.36	3.12
S.D.	4.26	2.88
Mean A v. B, $P > 0.05$		
NOT SYMPTOMATIC, NOT ATOPIC		(Group C)
No. of children		11
No. of assays		12
Maximum stimulation by antigen:		
Significant increase		9/11 (81%)
Range		0.73-4.34
Mean		2.12
S.D.		1.10
Mean A v. C, $P < 0.05$		
Mean B v. C, $P > 0.05$		

Table 4. Lymphocyte stimulation by soybean protein (skin test with soya protein not done§)

SYMPTOMATIC, ATOPIC	(Group A)
No. of children	22
No. of assays	22
Maximum stimulation by antigen:	
Significant increase	18/22 (81%)
Range	0.69-14.40
Mean	4.00
S.D.	3.40
NOT SYMPTOMATIC, NOT ATOPIC	(Group B)
No. of children	14
No. of assays	15
Maximum stimulation by antigen:	
Significant increase	13/14 (92%)
Range	1.05-3.34
Mean	1.94
S.D.	0.64
Mean A v. B, $P < 0.05$	

§ Solution of soybean protein prepared for skin testing gave irritant reactions in all normal persons tested and was considered unsuitable for use.

allergic children, with or without positive skin test to the food protein added to the cultures.

With wheat the mean value for lymphocyte stimulation was significantly greater in the symptomatic, atopic group with positive skin tests to wheat compared to the mean of the group not symptomatic, not atopic (Table 3). The same was true with soybean proteins but the groups could not be subdivided according to positive or negative skin tests due to skin irritation by the soybean extract (Table 4).

The findings in one child were so exceptional that the data were not included in the tabulation and analysis of Tables 1-4. This was an atopic child with asthma and eczema and positive skin tests with cow's milk proteins and ovalbumin. Because of the eczema, soybean formula had been substituted for milk in the diet. The child was also mentally retarded and had frequent periods of vomiting, during which he often aspirated the food ingested. During periods when either cow's milk, eggs or soybean formula were in the diet, and vomiting and aspiration were frequent, addition of the corresponding antigen to lymphocyte cultures gave the highest values for increased stimulation we observed: milk proteins, 13.0; ovalbumin, 8.7; and soybean protein, 50.2.

These findings suggested that aspiration of food into the lungs may promote greater immunologic response, and so a group characterized by frequent aspiration was studied, namely, children with dysautonomia. The effect of cow's milk proteins was determined on cultures of lymphocytes from five children, 2-12 years, with dysautonomia but not atopic. Skin tests with cow's milk proteins were negative. Values for maximum stimulation ranged from 1.31 to 13.59, the mean being 7.61 with S.D. 4.39. The mean of this study was significantly greater ($P < 0.02$) than the means of the symptomatic, atopic children, with or without a positive skin test to milk proteins (Table 1, A and B).

Antigenic release of histamine from leucocytes

The incidence and magnitude of histamine release from leucocytes of allergic children by the food proteins tested are given in Table 5. The thirty-five subjects were clearly allergic and gave definite positive skin tests with the food proteins tested, but their symptoms were not necessarily caused by the food proteins responsible for the positive

Table 5. Histamine released from leucocytes by food proteins in thirty-five allergic children*

Antigen	No. of tests	Released over 20% of histamine	Maximum release obtained (%)	Concn antigen required for max. release ($\mu\text{g/ml}$)
Milk proteins	19	10	27-70	20-2000
Ovalbumin	12	6	22-86	0.2-2000
Wheat extract	8	2	33-65	20-60

* The allergic subjects had immediate weal and flare reactions to intra-dermal injection with the food protein tested for histamine release and with other allergens, and also had definite clinical manifestations of allergic disorders—but not necessarily due to food.

skin tests. The data reveal a limited association between positive skin tests and leucocyte histamine release in the case of universally ingested food proteins. In our experience almost all children with positive skin tests to common inhalants such as ragweed, alternaria or house dust show more than 20% histamine release from their leucocytes with the corresponding allergen. We never found histamine release from leucocytes of children with negative intradermal skin tests to corresponding food proteins.

Discussion

Lymphocyte stimulation

These studies reveal that peripheral lymphocytes of nearly all children undergo significant stimulation upon *in vitro* culture with food proteins commonly consumed. The frequency of a significant stimulation was as great in non-allergic as in allergic children, whether a positive skin test with the food proteins was present or not. Furthermore, the average magnitude of the stimulation was not significantly different when lymphocytes from non-allergic and allergic children were exposed to proteins from foods almost universal in the diet, namely cow's milk and chicken eggs. In the case of soybean protein, the average of lymphocyte stimulation was significantly greater in atopic children, in whose diet soybean protein formulas are often substituted for cow's milk. The greatest stimulation of lymphocytes by food proteins was found in children with disorders causing frequent vomiting and aspiration of food into the lungs.

Formerly, antigenic stimulation of small lymphocytes to transform to large blast forms was believed to depend on direct action on the lymphocyte membrane without intervention of humoral antibodies or immunoglobulins. Therefore, antigenic lymphocyte stimulation was considered to reflect cellular immunity or the delayed type of hypersensitivity exclusively. Recently, specific immunoglobulins have been demonstrated on the surface of a small percentage of peripheral lymphocytes of animals (Sell & Gell, 1965) and man (Rabellino *et al.*, 1971). The presence of immunoglobulins on small lymphocytes provides a mechanism whereby specific combination with antigen could theoretically stimulate the reactions leading to transformation to blast forms.

Specific antibodies to cow's milk proteins appear in the serum of virtually all persons soon after cow's milk is introduced into the diet (Rothberg & Farr, 1965). These antibodies are predominantly of immunoglobulin class G (Kletter *et al.*, 1971). The frequent occurrence of antibodies in serum to protein of a food can be attributed to absorption of intact food protein from the gut (Ratner & Griehl, 1934), which would provide an antigenic stimulus to specific antibody production in any immunologically competent individual. Whether immediate or delayed symptoms arise when persons whose sera contain antibodies to a food protein are fed the food, depends on the kind of antibody and many other factors (Barrick & Farr, 1965). An atopic person is characterized by more ready production than others of enough IgE antibody to sensitize cells for immediate allergic reactions through specific antigenic histamine release.

From the foregoing one may view the uniformity in lymphocyte stimulation by food proteins in allergic and non-allergic children to be a natural consequence of the response of immunologically competent children to the absorption of intact protein from foods universally in the diet. When an unusual food like soybean protein is

consumed by allergic children more than others, stimulation of peripheral lymphocytes by that food protein may be expected to be greater in the group consuming soybean protein more frequently. Cross reactivity between proteins from soybean and other legumes (peas, beans) (Crawford *et al.*, 1965) or soybean hidden in food mixtures may account to some extent for lymphocyte stimulation in persons whose diets were considered free of soybean protein by history.

The exceptionally high values for lymphocyte stimulation by food proteins in children who frequently aspirate food into the lungs may be due to a greater efficiency of antibody production with introduction of antigen into the body by this route compared to the gut (Nelson, 1964; Druck *et al.*, 1969).

Test of lymphocyte stimulation by food protein in the manner used in the present study will not give definitive information on the role of a food suspected of causing symptoms in an allergic child. This does not mean that the demonstration of stimulation of lymphocytes by food proteins *in vitro* does not reveal an immunologic reaction with potential for pathogenesis of symptoms at times; many factors which may enter into the development of symptoms from an immunologic reaction like lymphocyte transformation will have to be clarified, as has been partly done for precipitation, haemagglutination and antigen-binding reactions (Rothberg & Farr, 1965; Barrick & Farr, 1965).

It should be mentioned that another study of lymphocyte culture as an aid in diagnosis of food allergy yielded results the authors believed to be 'of great value' (Amache, Ky & Hazard, 1969).

Histamine release

In our experience antigenic release of histamine is not obtained from leucocytes of persons with a negative intradermal skin test with the antigen. Usually, but not always, significant release is obtained with inhalant allergens when the skin test is positive. Significant specific histamine release was obtained less frequently with food proteins in skin-test positive children, but there was no release from leucocytes of non-allergic children. Our findings are in contrast to lack of histamine release from leucocytes of patients allergic to food reported by Dockhorn (1969), but in agreement with the findings of Galant, Frick & Bullock (1972).

Intradermal injection of food protein is a more sensitive but not more specific means of identifying immediate type of hypersensitivity than *in vitro* release of histamine from leucocytes. We cannot say from our study which may best predict allergic reactions to a food, but it is unlikely that any one currently available immunologic measurement will suffice for this purpose.

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