

Leukocyte migration inhibition test in children with cow milk allergy

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The usefulness of the leukocyte migration inhibition factor (LIF) test to detect cow milk (CM) hypersensitivity was studied in 40 children with suspected allergy to CM. Hypersensitivity was carefully investigated by oral milk challenges, which gave a final confirmation of cow milk hypersensitivity in 12 subjects, and excluded it in the remaining 28 subjects. Leukocyte migration inhibition was measured using β -lactoglobulin (BLG), α -lactalbumin (ALA), α -casein (ACA) and bovine serum albumin (BSA) as antigens. IgA and IgG antibodies to these antigens were measured by an enzyme-linked immunosorbent assay, and IgE antibodies to these antigens and to CM by radioallergosorbent test (RAST). Skin prick test with CM was performed in 38 subjects, and with BLG, ALA, ACA and BSA in 29 subjects. Leukocyte migration was more often inhibited by cow milk antigens in the CM challenge positive (CM+) subjects than in the challenge negative (CM-) subjects. Of the specific milk antigens, ALA was the most potent inhibitor, and gave a positive LIF test result in all CM+ subjects, and significantly ($P < 0.02$) less often (15/24) in CM- subjects. Also in the skin prick test and RAST, ALA gave positive results more often than the other milk antigens. BLG, ACA and BSA had an inhibiting effect on leukocyte migration, but the difference between the CM+ and CM- subjects was not statistically significant. Two of the 12 CM+ subjects had no demonstrable IgE antibodies to CM proteins; both of them, however, had a positive LIF test with at least one of the CM antigens. No significant correlation was observed between leukocyte migration inhibition and the serum levels of IgA, IgG or IgE antibodies to milk antigens. The results of this study suggest that cell-mediated immunity may play a role in cow milk allergy, also in subjects with demonstrable IgE antibodies to CM. Even though the LIF test is sensitive in the diagnosis of cow milk allergy, it is not useful as a diagnostic test, since many challenge-negative subjects had a positive LIF test.

Key words: α -casein; α -lactalbumin; β -lactoglobulin; bovine serum albumin; cell-mediated immune response; cow milk allergy; leukocyte migration inhibition factor.

Accepted for publication 1 May 1987.

The diagnosis of hypersensitivity to foods is a most difficult task for a clinician. A correct diagnosis is especially needed when the suspected food is difficult to avoid and/or of nutritional importance, as is the case with cow milk.

As hypersensitivity to cow milk (CM) is often of immediate type (15), IgE antibodies to milk as well as positive skin test reactions are often found in milk-sensitive subjects (6, 17). However, even unsymptomatic and CM-tolerant subjects may show positive skin test reactions or IgE antibodies to cow milk (3, 5, 16). As false

negative results of skin tests and RAST with CM have also been reported (7, 9, 12), other mechanisms than immediate, IgE-mediated allergy have been proposed to explain these reactions. The measurement of a cellular, leukocyte migration inhibiting, factor (LIF; 2) has been reported useful, particularly in the diagnosis of delayed-onset symptoms after milk ingestion(13).

To examine the usefulness of the LIF test in the diagnosis of CM hypersensitivity, we studied a group of subjects with suspected hypersen-

sitivity to cow milk in whom the clinical diagnosis was carefully confirmed by challenge procedures.

PATIENTS AND METHODS

Patients

We investigated 40 subjects, mainly small children, in whom CM-induced symptoms had been suspected by the parents or a physician. The age of the children varied from 4 months to 17 years ($\times \pm$ SD 30 ± 37 months, median 16 months).

The most common complaint associated with milk intake was the appearance or worsening of atopic eczema (73 %) and/or gastrointestinal symptoms (39 %). Other symptoms included urticaria (15 %), asthma (3 %), other respiratory symptoms (13 %), anaphylactic-like reactions (10 %), restlessness (5 %), and conjunctival symptoms (3 %). The symptoms were multiple in 18 cases (45 %).

Methods

After a 2 weeks' elimination diet a challenge with increasing amounts of CM was performed at home by the parents. If no symptoms occurred during 1 week's normal intake of milk, the child was considered tolerant to cow milk (13 subjects). In the remainder, an open challenge was performed in the hospital or outpatient clinic with increasing amounts of CM. If no symptoms appeared during 2 days' observation, the challenge was continued at home for at least 1 week. The hospital challenge was negative in six cases. Another six subjects reacted with anaphylaxis or severe urticaria after small amounts, and were considered intolerant to cow milk (CM+). The remaining cases underwent a double-blind challenge with CM. Cow milk was mixed with an appropriate liquid (e.g. soy or sesame seed formula if tolerated by the child) which resembled milk in appearance. The dose was increased gradually from 1 ml to 200 ml during 2 days, after which the challenge was continued at home for a further 3 days on a double-blind basis. The daily dose of CM varied from 300 to 500 ml. During

the control period the challenge protocol was identical, except that the milk-like liquid was used instead of CM. The control and CM periods were randomized. The challenge was considered positive if symptoms similar to those experienced at previous exposures were observed during the milk challenge, but not during the placebo period. The double-blind challenge gave a negative result in nine subjects, making a total of 28 cow milk tolerant subjects (CM-). A positive result was obtained in six subjects (immediate reactions in four, delayed in two), making a total of 12 cow milk intolerant subjects (CM+).

Skin prick tests were performed with milk as such and with purified α -lactalbumin (ALA; Sigma), β -lactoglobulin (BLG; Sigma), α -casein (ACA; Merck) and bovine serum albumin (BSA; Sigma) at a concentration of 1 mg/ml. The skin test results were evaluated according to the method recommended by the Northern Society of Allergology (1): a wheal size equal to the reaction of the positive control (histamine hydrochloride 1 mg/ml) was recorded as a 3+ reaction. Two subjects were not tested due to their lack of cooperation.

A blood sample was obtained from each subject at the time of the challenge procedures. Serum was stored at -20°C until analyzed. Serum IgE antibodies to CM, ALA, BLG and ACA were measured by commercial Phadebas RAST reagents using f2 (Pharmacia AB, Uppsala, Sweden), f76, f77 and f78 (MIAB Inc., Knivsta, Sweden) allergen discs, respectively. For the measurement of IgE antibodies to BSA, experimental allergen discs provided by Pharmacia were used. The RAST results were expressed as a score from 0 to 4 according to the scale given by the manufacturer, or as percentage of the total activity.

IgA and IgG antibodies to ALA, BLG, ACA and BSA were measured by ELISA (18). The results were expressed as optical density units.

To evaluate the production of LIF after stimulation with specific antigen *in vitro* an indirect (14) method was applied. Mononuclear cells were separated from heparinized peripheral blood by Ficoll-Isopaque centrifugation (4). One million cells in 0.9 ml of RPMI

1640 supplemented with 50 µg/ml gentamycin, 10% AB serum (The Finnish Red Cross Laboratory, Helsinki, Finland) and 0.2% sodium bicarbonate were incubated for 2 h in 12 × 75 mm, round-bottomed tissue culture tubes (Falcon 2058, Falcon, Oxnard, CA) together with 100 µl of either plain medium (RPMI) or the control mitogens (PHA 0.2 mg/ml; Difco Laboratories, and PPD 10 µg/ml; Statens Seruminstitut, Denmark) or with the specific allergens (ALA, BLG, ACA and BSA) at a concentration of 10 µg/ml. The cultures were set up in duplicate. After a 2-h induction phase the cells were washed three times with RPMI 1640, the supernatant was discarded and 0.8 ml of fresh RPMI 1640 supplemented with AB serum and gentamycin was added. The cells in each tube were mixed and pipetted in a volume of 200 µl as triplicates into U-shaped wells of tissue culture plates (Linbro, Titertek, Flow Laboratories, Inc. Hamden, CT). After 24-h incubation at 37°C in humidified air and 5% CO₂ the supernatants were collected, pooled, and stored at -70°C in two portions until the LIF activity was determined.

LIF activity was measured using mouse spleen cells as targets. Spleen cells were isolated from young 6–8 week old mice by homogenizing the spleens through a metallic mesh. The cells were washed RPMI 1640 supplemented with 15% of fetal calf serum (Gibco Biocult, Glasgow, Scotland) and adjusted to 100 to 150 million cells/ml. Hematocrit capillaries (Medlab, Poland) were filled with cells and one edge was closed with a paraffin-vaseline mixture, and then the cells were spun down (15 min at 4°C at 400 g). The capillaries were cut at the level of buffy coat and each was placed at the bottom of a well of a Costar 3524 plate (Costar, Cambridge, Mass.) with crease. Thereafter, 600 µl of the supernatant to be tested was added to the wells for 18 h incubation at 37°C. Migration of the leucocytes out of the capillaries was measured using a Nikon SMZ-1 inverted microscope. Migration indices (MI) of the duplicate measurements were calculated as follows:

$$MI = \frac{\text{area migrated in the presence of antigen/mitogen}}{\text{area migrated in the absence of antigen/mitogen}}$$

MI values < 0.80 were considered positive.

The sensitivity, specificity, and overall test efficiency of the LIF test were calculated as described by Galen & Gambino (8). The statistical significance of the differences between the two means was tested by using Student's t-test. The statistical significance of the differences between the observed grouped distributions was tested by means of the chi-square test.

RESULTS

A positive skin prick test (SPT) results with fresh milk was obtained in all (11/11) milk challenge positive (CM+) cases (Table 1), and in three of 27 challenge negative (CM-) cases.

A positive (score 1–4) RAST with CM was observed in 10 of 12 CM+ subjects, and in eight of 27 CM- subjects (Table 1).

Of the specific milk proteins, a positive SPT and a positive RAST result were most often observed with α-lactalbumin (Table 1). All of the 12 milk challenge positive (CM+) subjects (Table 2), and only 15 of 24 challenge negative (CM-) subjects ($P < 0.02$) had a positive LIF test with ALA. The migration index (MI) with ALA was lower ($P < 0.02$) in CM+ subjects ($\bar{x} \pm SD = 0.60 \pm 0.13$) than in CM- subjects ($\bar{x} \pm SD = 0.71 \pm 0.15$).

The MI with β-lactoglobulin (BLG) was lower in CM+ subjects ($\bar{x} \pm SD = 0.62 \pm 0.18$) than in the CM- subjects ($\bar{x} \pm SD = 0.70 \pm 0.15$), but the difference was not statistically significant. A positive LIF with BLG was obtained in 9/12 of the CM+ subjects, and in 17/28 (61%) of the CM- subjects ($P > 0.1$; Table 2).

Leukocyte migration was slightly more often inhibited by ACA in the CM+ subjects than in the CM- subjects, but the difference was not statistically significant. No difference was observed in the LIF test with BSA between the CM+ and CM- subjects (Table 2).

The two CM+ subjects with a negative cow

milk RAST (patients No. 6 and 11; Table 1) developed their symptoms in the double-blind-challenge slowly, within a few days. Both of them responded to the double-blind milk challenge with gastrointestinal symptoms and rhinorrhea. They had a positive LIF result with at least one of the antigens tested (Table 2).

No significant correlations between the leukocyte migration index and the IgE (Fig. 1), IgA or IgG antibodies to the different CM proteins were found. The migration indices with ALA and BLG had a negative correlation with the age of the patients ($r = -0.784$ and -0.648 , respectively). In our study population the sensitivity of the

Table 1
Clinical and allergological characteristics of the patients

Patient	CM challenge	Sex	Age (mo)	Skin prick test					RAST (score)				
				CM	BLG	ALA	ACA	BSA	CM	BLG	ALA	ACA	BSA
1	+	M	9	4	0	3	3	4	3	2	3	3	3
2	+	M	5	3	3	3	1	0	3	2	4	0	0
3	+	M	8	3	3	3	3	1	3	1	4	1	0
4	+	M	16	4	4	4	2	1	1	0	1	0	0
5	+	M	30	1	0	0	4	0	1	0	0	0	0
6	+	M	16	nd	nd	nd	nd	nd	0	0	0	0	0
7	+	M	19	6	4	5	4	4	4	4	4	3	2
8	+	F	23	3	2	3	1	3	2	2	2	0	0
9	+	F	13	4	0	4	0	0	1	0	2	0	0
10	+	M	9	6	5	4	3	1	3	2	3	0	0
11	+	F	204	3	0	1	2	2	0	0	0	0	0
12	+	M	15	6	4	0	4	4	3	4	4	4	3
13	-	M	16	0	0	0	0	0	2	1	0	0	0
14	-	M	70	0	0	0	1	0	0	0	0	0	0
15	-	M	18	0	0	0	0	0	0	0	0	0	0
16	-	F	29	0	0	0	0	0	0	0	0	0	0
17	-	M	8	0	nd	nd	nd	nd	0	0	0	0	0
18	-	F	26	0	0	0	0	0	0	0	0	0	0
19	-	F	14	0	0	0	3	0	1	0	0	1	0
20	-	F	4	0	0	0	0	0	2	2	2	2	0
21	-	M	16	4	0	0	0	0	1	0	0	0	0
22	-	M	26	0	nd	nd	nd	nd	0	0	0	0	0
23	-	M	62	0	nd	nd	nd	nd	0	0	0	0	0
24	-	M	21	2	nd	nd	nd	nd	3	0	1	2	0
25	-	F	16	0	0	0	0	0	0	0	0	0	0
26	-	F	27	0	0	0	0	0	1	2	2	0	2
27	-	F	6	0	0	0	0	0	0	0	0	0	0
28	-	M	26	0	nd	nd	nd	nd	0	0	0	0	0
29	-	M	19	0	0	0	0	0	0	0	0	0	0
30	-	F	16	0	0	0	0	0	0	0	0	0	0
31	-	M	10	6	1	3	4	4	3	1	0	3	1
32	-	M	26	0	0	0	0	0	0	0	0	0	0
33	-	M	78	0	nd	nd	nd	nd	0	0	0	0	0
34	-	M	13	0	0	0	0	0	0	0	0	0	0
35	-	M	98	0	nd	nd	nd	nd	0	0	0	0	0
36	-	F	10	nd	nd	nd	nd	nd	0	0	0	0	0
37	-	F	116	0	nd	nd	nd	nd	0	0	0	0	0
38	-	M	41	0	nd	nd	nd	nd	0	0	0	0	0
39	-	F	10	0	0	2	0	1	0	0	1	0	2
40	-	M	17	0	0	0	0	0	1	2	0	0	0

Abbreviations: ALA = α-lactalbumin; ACA = α-casein, BLG = β-lactoglobulin; BSA = bovine serum albumin; CM = cow milk; nd = not done

LIF test in the diagnosis of intolerant subjects varied from 100 % (ALA) to 50 % (BSA) depending on the antigen. The specificity of the LIF test, however, was low: from 33 % (ACA) to 46 % (BSA). Its specificity would naturally be higher if the criterion value of the positive migration index were increased to, e.g. 0.60, but this would make

the test less sensitive and thus not significantly change its overall efficiency.

DISCUSSION

The subjects in this study were a population of children referred to our clinic for suspected cow

Table 2
LIF test results and cow milk (CM) challenge*

Patient	CM challenge	Leukocyte migration inhibition factor (LIF)					
		PHA	PPD	ALA	BLG	ACA	BSA
1	+	0.53	0.53	0.66	0.53	0.20	0.16
2	+	0.74	0.64	0.63	0.67	0.85	0.83
3	+	0.39	0.28	0.38	0.35	0.25	0.25
4	+	0.56	0.61	0.45	0.36	0.67	0.81
5	+	0.71	0.68	0.70	0.72	0.70	0.69
6	+	0.68	0.70	0.67	0.80	0.82	0.84
7	+	0.10	0.52	0.67	0.80	0.74	0.72
8	+	0.41	0.29	0.35	0.38	0.69	0.72
9	+	0.50	0.70	0.70	0.63	0.70	0.78
10	+	0.71	0.64	0.70	0.62	0.90	0.82
11	+	0.67	0.62	0.78	0.88	0.56	0.84
12	+	0.16	0.52	0.56	0.64	0.71	0.83
13	-	0.71	0.65	0.65	0.72	0.82	0.81
14	-	0.61	0.73	nd	0.80	nd	0.68
15	-	0.49	0.70	0.75	0.72	0.80	0.68
16	-	0.27	0.26	0.30	0.52	0.52	0.35
17	-	0.38	0.67	0.67	0.44	0.40	0.41
18	-	0.40	nd	0.70	0.79	0.75	0.72
19	-	0.70	0.73	0.90	0.70	0.75	0.72
20	-	0.70	0.62	nd	0.51	nd	0.59
21	-	0.42	0.40	0.89	0.88	0.65	0.90
22	-	0.70	0.61	nd	0.83	nd	0.88
23	-	0.50	nd	0.85	0.85	0.79	0.89
24	-	0.56	0.60	0.80	0.72	0.70	0.82
25	-	0.72	0.70	0.70	0.80	0.80	0.82
26	-	0.70	0.60	0.92	0.80	0.70	0.75
27	-	0.48	0.72	0.88	0.69	0.92	0.68
28	-	nd	0.82	nd	0.83	nd	0.80
29	-	0.75	0.68	0.68	0.72	0.78	0.82
30	-	0.24	0.30	0.54	0.33	0.57	0.83
31	-	0.72	0.49	0.82	0.79	0.80	0.85
32	-	0.90	0.60	0.58	0.58	0.60	0.76
33	-	0.68	0.70	0.70	0.80	0.80	0.82
34	-	0.62	0.59	0.79	0.81	0.70	0.72
35	-	0.46	0.40	0.49	0.48	0.76	0.79
36	-	0.63	0.52	0.63	0.83	0.79	0.80
37	-	0.36	0.49	0.49	0.44	0.49	0.69
38	-	0.78	0.65	0.82	0.84	0.81	0.79
39	-	0.56	0.67	0.84	0.67	0.84	0.84
40	-	0.62	0.70	0.65	0.67	0.70	0.65

Abbreviations: PHA phytohemagglutinin; PPD = purified protein derivative; ALA, BLG, ACA, and BSA, see Table 1.
*LIF migration index (MI) values < 0.80 are considered positive.

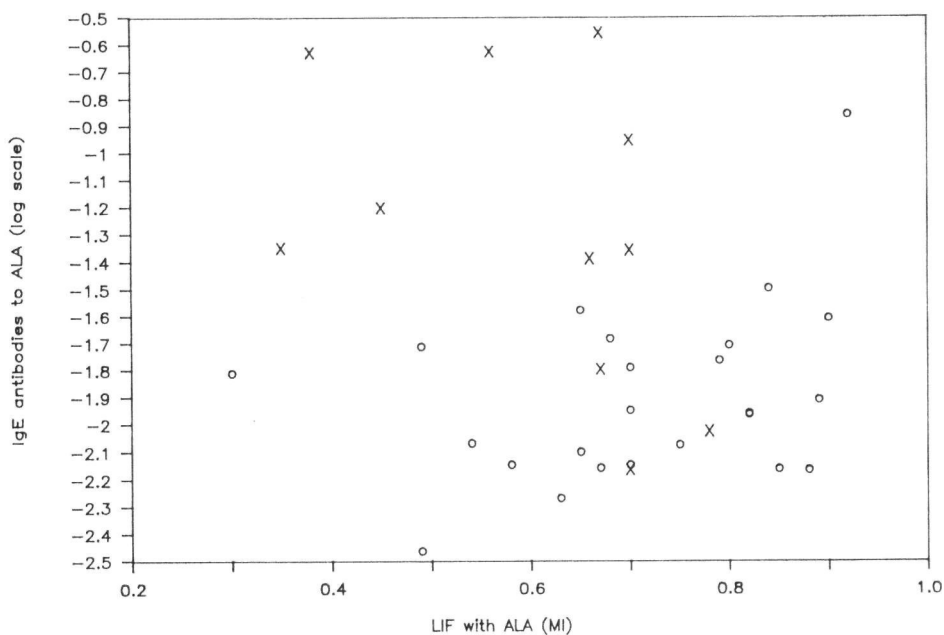


Fig. 1. No significant correlation was observed between leukocyte migration inhibition with α -lactalbumin (ALA) and the IgE antibody levels to ALA. Migration index (MI) values < 0.80 are considered positive. IgE antibody levels are expressed as a logarithm of the percentage of the total activity (cmp %). $\times$ = subjects positive to a challenge with milk; $\circ$ = subjects negative to challenge.

milk intolerance; to our knowledge they were not selected because of a specific symptom or a previous test result. The symptoms and the appearance of positive RAST results to CM in our study population were strikingly similar to the figures presented in a study from Sweden (5) indicating that our patients represent a patient material similar to that referred for CM allergy evaluation in other Nordic countries.

In our study, α -lactalbumin gave the highest number of positive results in the RAST and in the LIF test. Previously, Goldman et al. (10) have shown that in milk-allergic children, casein, α -lactalbumin, β -lactoglobulin and bovine serum albumin gave positive reactions with fairly equal frequency (from 52% with BSA to 68% with BLG), when challenged orally with doses present in 100 ml skim milk. In the skin test (11), when equal concentrations of each purified protein were used, Goldman et al. observed most positive reactions with ACA (42%) and least with BLG (29%), as also found in our study. In both studies, however, the observed differences in the frequencies of positive reactions in the skin tests with the different proteins are not statistically significant.

A positive LIF test result could be a sign of a

non-IgE-mediated allergy to a food. In our study population, however, all challenge positive subjects with a positive RAST and/or skin test also had a positive LIF test. This may indicate that a cell-mediated immune response is an important prerequisite for CM allergy, but that without a simultaneous IgE-response clinically important hypersensitivity symptoms seldom occur.

The number of LIF positive subjects who clinically tolerated cow milk was relatively high. We cannot exclude the possibility that at least some of these subjects may have previously been allergic to cow milk, but recovered from their hypersensitivity state before the challenges were performed. Ashkenazi et al. (2), however, have previously reported that LIF results were within normal range (> 0.80) in such children. None of the previous reports on the LIF test in the diagnosis of CM allergy have included a group of allergic children not reacting to cow milk. Many of the CM tolerant children in the present study had atopic eczema, and most of them (85%) were reported to react to other foods, e.g. egg, fish, tomato, etc. This may have led to increased permeability of the intestinal tract, allowing larger amounts of

milk antigens to gain access to the gut-associated immune system. Thus an abnormal LIF test result to food allergens may reflect an increased absorption of food antigens rather than allergy to a specific antigen. The LIF test was shown to be a sensitive marker of an immune response, but not specific enough for diagnostic purposes. In CM allergic patients the skin prick test, and to a lesser degree, the RAST, were more specific than LIF.

ACKNOWLEDGEMENTS

We wish to thank Ms. Marjo Ingman and Ms. Raija Raulimo for their skilful technical assistance. This work was supported by the Finnish Allergy Research Foundation, Piltti Foundation and by a grant to E.S. from the Finnish Academy.

REFERENCES

1. Aas, K.: Standardization of allergen extracts. Standardization of diagnostic work in allergy. *Clin. Allergy* 3, 481-482, 1973.
2. Ashkenazi, A., Levin, S., Idar, D., Or, A., Rosenberg, I. & Handzel, Z. T.: In vitro cell-mediated immunologic assay for cow's milk allergy. *Pediatrics* 66, 399-401, 1980.
3. Björkstén, F. & Saarinen, U. M.: IgE antibodies to cow's milk in infants fed breast milk and milk formulae. *Lancet* 2, 624-625, 1978.
4. Böym, A.: Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. *Scand. J. Clin. Lab. Invest.* 21, Suppl. 97, 77-89, 1986.
5. Dannaeus, A. & Inganäs, M.: A follow-up study of children with food allergy. Clinical course in relation to serum IgE- and IgG-antibody levels to milk, egg and fish. *Clin. Allergy* 11, 533-539, 1981.
6. Dannaeus, A. & Johansson, S. G. O.: A follow-up study of infants with adverse reactions and RAST in relation to clinical course. *Acta Paed. Scand.* 68, 377-382, 1979.
7. Galant, S. P., Bullock, J. & Frick, O.: An immunological approach to the diagnosis of food sensitivity. *Clin. Allergy* 3, 363-372, 1973.
8. Galen, R. S. & Gambino, S. R.: Beyond normality: The predictive value and efficiency of medical diagnoses, pp. 10-40. John Wiley, New York, 1975.
9. Gavani, U. D., Hyde, J. S. & Moore, B. S.: Hypersensitivity to milk and egg white. Skin tests, RAST results and clinical intolerance. *Ann. Allergy* 40, 314-318, 1978.
10. Goldman, A. S., Anderson, D. W., Sellers, W. A., et al.: Milk allergy. I. Oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics* 32, 425-443, 1963.
11. Goldman, A. S., Sellers, W. A., Halpern, S. R., et al.: Milk allergy. II. Skin testing of allergic and normal children with purified milk proteins. *Pediatrics* 32, 572-579, 1963.
12. Hill, D. J., Davidson, G. P., Cameron, D. J. S. & Barnes, G. L. The spectrum of cow's milk allergy in childhood. *Acta Paediatr. Scand.* 68, 847-852, 1979.
13. Minor, J. D., Tolber, S. G. & Frick, O. L.: Leukocyte inhibition factor in delayed-onset food allergy. *J. Allergy Clin. Immunol.* 66, 314-321, 1980.
14. Räsänen, L., Karhumäki, E. & Krohn, K.: Cellular cooperation in mitogen-induced human leukocyte inhibitory factor (LIF) synthesis. *Cell. Immunol.* 44, 395-403, 1979.
15. Savilahti, E.: Cow's milk allergy. *Allergy* 36, 73-88, 1981.
16. Van Asperen, P. P., Kemp, A. S. & Mellis, C. M. Skin test reactivity and clinical allergen sensitivity in infancy. *J. Allergy Clin. Immunol.* 73, 381-386, 1984.
17. Vanto, T., Kalimo, K. & Viander, M.: Diagnosis of hypersensitivity to cow's milk (abstract). *Ann. Allergy* 55, (part II):383, 1985.
18. Vanto, T., Viander, M. & Koivikko, A. Humoral and cell mediated immune response to dog dander and hair in asthmatic children. *Allergy* 38, 103-112, 1983.

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