

COQ
31325

In vitro Translation of Messenger RNA from the House Dust Mite *Dermatophagoides pteronyssinus*

G.A. Stewart, W.R. Thomas

Clinical Immunology Research Unit, Princess Margaret Hospital, Subiaco, Western Australia

Abstract. RNA was extracted from the house dust mite *Dermatophagoides pteronyssinus* and a polyadenylated [poly(A)⁺] and non-polyadenylated [poly(A)⁻] mRNA isolated by oligothymidylic acid-cellulose chromatography. Both preparations were translated in vitro using a cell-free rabbit reticulocyte system. The relative incorporation of ³⁵S-methionine into trichloroacetic acid-precipitable translation products obtained using poly(A)⁺ and poly(A)⁻ mRNA was 21- and 1.3-fold, respectively, when compared with control translations performed without added mRNA. Sodium dodecylsulphate-polyacrylamide gel electrophoretic analysis of the translation products in combination with autoradiography showed that many proteins with apparent molecular weights in the range 14,000->67,000 daltons were synthesized. Immunoprecipitation studies performed using a sheep anti-whole mite antiserum showed that several of the synthesized proteins corresponded with mite antigens. These findings were confirmed by coprecipitation studies using crossed immunoelectrophoresis (CIE). A comparison of the CIE data with those obtained with crossed radio-immunoelectrophoresis showed that 6 of the 9 allergens previously described in our laboratory were synthesized, namely *Der p* I, Dpt 1, 3, 4, 8 and 17. The allergenicity of the lysates was also confirmed by immunoprecipitation studies using a specific anti-human IgE reagent.

Introduction

Allergens from the house dust mite *Dermatophagoides pteronyssinus* are major triggers of allergic disease such as asthma and perennial rhinitis [1, 2]. Because of this many studies have been devoted to their isolation and characterization. In particular, such studies have been undertaken to gain insight into the factors influencing the aetiology of mite-induced allergic disease, to develop standardized diagnostic reagents, and ultimately to optimize immunotherapeutic procedures.

However, although several groups have shown that mite extracts contain several major and minor allergens [3-5], the number of mite allergens that have been purified in sufficient quantity is limited, due in no small part to the complexity of mite extracts. For example, not only are some of the major allergens quantitatively minor constituents of mite extracts, but

also the number of mite allergens in crude extracts, as judged by techniques such as crossed immunoelectrophoresis (CIE) and Western blotting analysis, is relatively high. With the advent of recombinant DNA technology, however, the production of some or all of the important mite allergens in sufficient quantity for study without recourse to complex physicochemical purification procedures is now feasible.

Given that the production of IgE is highly T-cell-dependent [6] and that T cell responses are directed towards protein antigens which can be processed by the lysosomal system of antigen-presenting cells [7], it is highly probable that at least parts of the mite allergens are protein and could, therefore, be translated from cDNA cloned into *Escherichia coli*. It is not, however, clear that mRNA isolated from whole mites would be a suitable starting point for this procedure. This paper shows that polyadenylated [poly(A)⁺] RNA isolated from whole mite homogenates codes

for many mite proteins which can be readily detected after in vitro translation and that these include the major faecal allergen *Der p I* [8].

Materials and Methods

Mites

Mites were obtained from Commonwealth Serum Laboratories (Parkville, Australia). The mites were harvested by the manufacturer and flown overnight to Perth at ambient temperature in a glass bottle without nutrient. Before use, they were checked for viability by microscopy.

Preparation of mRNA

30 ml of 6 mol/l guanidine hydrochloride (BRL, Gaithersburg, USA) in 0.1 mol/l sodium acetate, pH 5.2, were added to 5 g of live mites and the suspension immediately homogenized. The soluble mite material was clarified by centrifugation at 10,000 rpm for 30 min in a Beckman 50 Ti rotor, and the supernatant was then centrifuged through a 4.8 mol/l CsCl pad containing 10 mmol/l EDTA at 35,000 rpm in a Beckman SW41 rotor. The pelleted RNA was fractionated on oligo(dT) cellulose (Collaboration Research, Lexington, USA) as previously described [9], and the bound RNA [poly(A)⁺] and the unbound (non-polyadenylated) RNA [poly(A)⁻] were isolated. The poly(A)⁺ fraction underwent a further 2 cycles of purification.

In vitro Translation

Mite mRNA was translated using a rabbit reticulocyte lysate system (N90, Amersham International, Amersham, UK), according to the manufacturer's instruction. The reaction mixture contained 10 µl lysate, 0.5 µg mRNA and 20 µCi ³⁵S-methionine. Reactions were allowed to proceed at 30°C and stopped after 60 min incubation. Aliquots were applied to GFA filters (Whatman, Maidstone, UK) and incubated on ice for 15 min in 5% w/v trichloroacetic acid (TCA) containing 1 mg/ml methionine, and then boiled in this solution for 15 min. The filters were washed and the precipitated radioactivity was counted by liquid scintillation.

Preparation of Mite Extracts

The mites were extracted by grinding at 0°C in 0.05 mol/l of ammonium bicarbonate, pH 8.0, and the resulting extract was clarified by centrifugation at 15,000 g for 15 min. The supernatant was removed and the insoluble material was extracted twice more in a similar manner. The supernatants were combined and dialyzed exhaustively against 0.05 mol/l of ammonium bicarbonate, pH 8.0. The supernatant was then freeze-dried and stored dessicated at -20°C until required.

The allergen *Der p I* was isolated by chromatofocusing from spent growth medium in a manner similar to that described earlier [10].

Antisera

The anti-whole mite extract and the anti-*Der p I* antisera were raised in sheep and rabbits, respectively, as described previously [3]. Briefly, 3 sheep were each immunized subcutaneously with 2 mg of whole mite extract. The extract was dissolved in 1 ml of

0.01 mol/l phosphate buffer, pH 7.2, containing 0.15 mol/l NaCl (PBS) and emulsified with Freund's complete adjuvant. A secondary injection of extract (2 mg) in Freund's incomplete adjuvant was given 28 days later and the sheep were bled 10 days after this and subsequent booster injections. Sera from each sheep were pooled and stored frozen at -20°C until required.

The anti-*Der p I* antiserum was raised in rabbits. Each of 3 rabbits was immunized subcutaneously with 500 µg of purified *Der p I* in 0.5 ml of PBS emulsified with an equal volume of Freund's complete adjuvant. The rabbits were boosted 26 days later with the same amount of material emulsified in Freund's incomplete adjuvant and bled 10 days after this and subsequent booster injections. Sera from each rabbit were pooled and stored at -20°C until required. The rabbit anti-human IgE and the sheep anti-rabbit IgG antisera were raised against IgE and IgG isolated by a combination of ammonium sulphate precipitation and diethylaminoethyl cellulose ion-exchange chromatography, as described previously [11].

Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli [12] using 7.5–18% w/v gradient polyacrylamide gels. The molecular weight markers used were obtained from Sigma Chemical Co., Ltd. (St. Louis, Mo.) and comprised albumin (molecular weight 67,000 daltons), ovalbumin (43,000 daltons), glyceraldehyde-3-phosphate dehydrogenase (36,000 daltons), carbonic anhydrase (29,000 daltons), trypsinogen (24,000 daltons), soy bean trypsin inhibitor (20,100 daltons) and α-lactalbumin (14,400 daltons). After electrophoresis, gels were stained with 0.06% w/v Coomassie blue R250. Autoradiography of gels containing ³⁵S-methionine-labelled translation products was performed after treating the destained gels with Amplify (Amersham International) according to the manufacturer's instructions.

CIE

Translated mite proteins were analyzed by CIE using a sheep anti-whole mite antiserum as described previously [3], with the following modifications. Prior to electrophoresis, 5 µl of whole mite extract (50 mg/ml) were incubated with 6 µl PBS containing 0.3 mg/ml methionine and 1 mg/ml ovalbumin for 30 min at room temperature. After incubation, the ³⁵S-methionine-labelled translation mixture (200,000 TCA-precipitable dpm) or translation control (i.e. no added mRNA) was added to the methionine-ovalbumin-mite mixture and electrophoresis was performed. After the second electrophoretic step, the gels were pressed and then washed (three times) in PBS containing 0.3 mg/ml methionine. After a further wash in distilled water, the CIE plates were dried and autoradiography performed using preflashed Curix RPI film (Agfa Gevaert, Belgium). CIE experiments were performed on three different occasions. Crossed radio-immunoelectrophoresis (CRIE) was performed as described previously using mite-atopic and non-atopic human serum pools [3]. In both CIE and CRIE, the antigen-numbering system described by Stewart et al. [3] was used.

Immunoprecipitation of Labelled Mite Proteins

³⁵S-methionine-labelled mite proteins were precipitated using specific anti-immunoglobulin and specific anti-mite antibodies in a double antibody system. To determine the spectrum of the translated mite proteins, a suitable aliquot of lysate (20,000–200,000 dpm) was added to 100 µl of sheep anti-whole mite

extract diluted 1:80 with 0.05 mol/l Tris buffer, pH 7.4, containing 0.15 mol/l NaCl and 0.5% v/v Triton-X 100 (TNT). After incubation for 4 h at room temperature, the sheep IgG was precipitated by incubation with an optimal (previously determined by quantitative precipitation) amount of rabbit anti-sheep IgG overnight at 4°C. The precipitate was then washed (three times) with TNT buffer, re-dissolved in SDS sample preparation buffer and heated at 100°C for 5 min prior to SDS-PAGE analysis. To detect putative ^{35}S -methionine-labelled *Der p I* protein, lysates were incubated with

rabbit anti-*Der p I* antiserum diluted 1:80 and treated as above, except that a sheep anti-rabbit IgG antiserum was used to precipitate the immunoglobulin. Control experiments were performed using lysates in combination with normal rabbit and sheep sera.

Immunoprecipitation of labelled mite proteins demonstrating reactivity with mite-specific IgE from human atopic donors was performed using a triple antibody assay as described previously [11]. Briefly, human sera were incubated with ^{35}S -methionine-labelled proteins for 4 h at room temperature. Rabbit anti-human

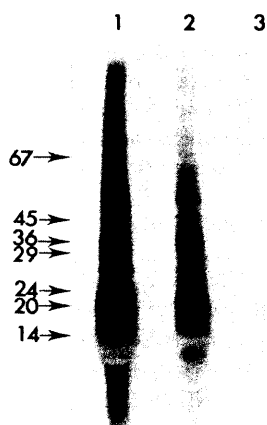


Fig. 1. Autoradiography of SDS-PAGE analysis of ^{35}S -methionine-incorporated mite proteins (lanes 1 and 2) and a control reticulocyte lysate (lane 3). Lanes 1 and 2 show proteins produced from two separate batches of mite mRNA. The apparent molecular weights ($\times 10^{-3}$) of the markers used are indicated on the left hand side of the autoradiograph.

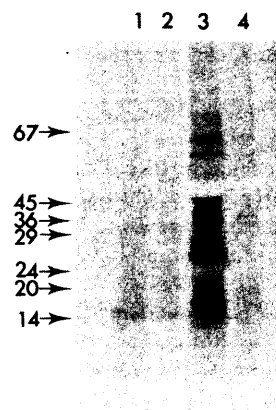


Fig. 2. Autoradiograph of SDS-PAGE analysis of ^{35}S -methionine-incorporated mite proteins precipitated using rabbit anti-*Der p I* antiserum (lane 1), normal rabbit serum (lane 2), sheep anti-whole mite extract (lane 3) and normal sheep serum (lane 4).

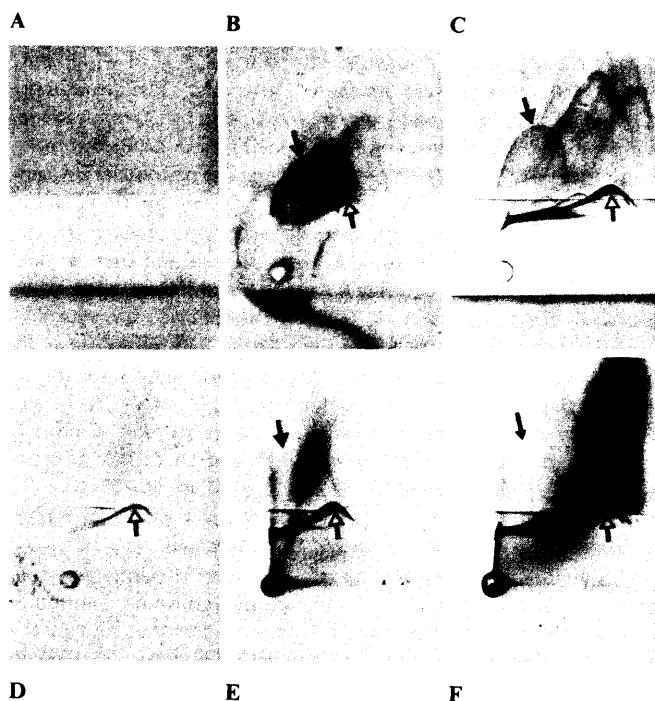


Fig. 3. Coprecipitation of ^{35}S -methionine-incorporated mite proteins with whole mite extract proteins in a CIE analysis. Panels A and B represent CIE analyses of mite allergens using non-mite-allergic (A) and mite-allergic (B) serum pools. Panel C shows a CIE profile stained for protein. Panels D, E and F represent autoradiographs obtained using non-mite RNA-containing (D) and mite RNA-containing lysates (E and F). In F, the amount of whole mite extract applied was twice that in E and the first-dimension electrophoretic step was extended to help resolve the complex banding pattern obtained in the middle of the autoradiograph. CIE was performed with an antiserum concentration of 52 $\mu\text{l}/\text{cm}^2$. The positions of *Der p I* ($\blacktriangle$) and *Dpt 4* ($\triangle$) are indicated by arrows.

IgE was then added and the mixture incubated for a further 2 h. The rabbit anti-IgE-mite-protein complex was precipitated with an optimal volume of sheep anti-rabbit IgG. The precipitate was washed and redissolved in 1 mol/l sodium hydroxide and the radioactivity bound was determined by liquid scintillation. The human sera used were screened for reactivity to mite allergens prior to the triple antibody assay by radioallergosorbent test (RAST) as described previously [11].

Results

In vitro Translation of Mite mRNA

mRNA was isolated from mites and translated in an in vitro translation system. It can be seen from table I that the greatest translation, as judged by the incorporation of ^{35}S -methionine, was obtained with

Table I. Translation of mite RNA in a cell-free rabbit reticulocyte system

RNA	Incorporation of ^{35}S -methionine at	
	0 min	60 min
Poly(A) ⁺	7,322	203,258
Poly(A) ⁻	7,610	13,250
No added RNA	6,241	9,821

The values given represent the mean (of two experiments) TCA-precipitable counts/minute obtained with 1 μg of poly(A)⁺ and 10 μg of poly(A)⁻ mite RNA.

poly(A)⁺ mRNA, where an approximately 21-fold increase in label incorporation over control (no added mRNA) was achieved. In contrast, a 1.3-fold increase in incorporation of label was obtained when poly(A)⁻ mRNA was translated (table I).

SDS-PAGE Analysis of ^{35}S -Methionine-Labelled Mite Proteins

An SDS-PAGE analysis of the mite proteins is shown in figure 1 and compared with the products of a control translation. Many mite proteins were produced with apparent molecular weights ranging from about 14,000 to > 67,000 daltons, with the majority in the region 14,000–60,000 daltons.

Immunoprecipitation of Labelled Mite Proteins

Mite proteins were immunoprecipitated with sheep anti-whole mite and rabbit anti-*Der p* I antisera, and analyzed by SDS-PAGE (fig. 2). At least 12 proteins were precipitated with the sheep anti-whole mite antiserum, the majority of which had molecular weights in the region of 14,000–45,000 daltons. Only faint, indistinct radiostaining was obtained with normal sheep serum. The rabbit anti-*Der p* I antiserum did not precipitate any distinct mite proteins.

Reactivity of Labelled Mite Proteins with Sera from Mite-Atopic and Non-Atopic Donors

The allergenicity of labelled proteins was assessed using appropriate sera in a triple antibody assay.

Table II. Reactivity of ^{35}S -methionine-labelled mite proteins with IgE from mite-atopic and non-atopic donors in a triple antibody assay

Serum No.	Mite allergy status	Total IgE U/ml	Mite RAST % radiation bound	Labelled mite protein precipitated cpm
0020	atopic	1,149	35.8	4,506
0242	atopic	436	37.8	1,953
0268	atopic	94	39.2	2,164
0359	atopic	1,175	43.2	1,981
0733	atopic	840	43.0	1,181
0988	atopic	1,500	38.6	1,897
0012	non-atopic	101	1.7	1,102
0296	non-atopic	12	1.5	881
0310	non-atopic	10	1.7	1,313
0748	non-atopic	553	1.8	1,045

Geometric mean counts/minute precipitated with atopic and non-atopic sera were 2,094 and 1,073, respectively ($p = 0.019$). The cpm values given are those obtained after deduction of non-specifically bound radioactivity obtained after incubating sera with an equivalent amount (cpm) of non-mite mRNA-containing translation mixture.

The results are shown in table II. The geometric mean levels of counts/minute precipitated with the atopic and non-atopic sera were 2,094 and 1,073, respectively. Analysis of the data by Student's *t* test showed that the means were significantly different ($p = 0.019$).

CIE Analysis of Mite Proteins

The translated mite proteins were examined by CIE, and the results obtained are shown in figure 3. Coprecipitation of labelled proteins with unlabelled carrier mite proteins was obtained and at least 14 proteins were detected (fig. 3E, F). The greatest radiostaining was obtained with the lipoprotein antigen Dpt 4 [3]. In contrast, radiostaining of *Der p* I was very weak. Experiments performed using a control translation mixture showed (fig. 3D) that slight non-specific staining was obtained with 3 antigens but predominantly with antigen Dpt 4. A comparison of the CIE data with those obtained from CRIE experiments (fig. 3A, B) showed that some of the antigens corresponded with allergens detected using a mite-allergic serum pool. In particular, the allergens *Der p* I, Dpt 1, 3, 4, 8 and 17 were identified. A CIE plate stained for protein is shown in figure 3C.

Discussion

Poly(A)⁺ and poly(A)⁻ RNA was isolated from the house dust mite by oligo(dT) cellulose chromatography, and the capacity of these two preparations to initiate translation in a reticulocyte system was studied. The most efficient fraction was found to be poly(A)⁺ RNA and the translation products obtained using it were analyzed to determine whether the resulting ³⁵S-methionine-labelled proteins were mite-specific.

Immunochemical analyses showed that mite-specific proteins were synthesized and, more importantly, that some were judged to be allergenic on the basis of their reactivity in an IgE-specific triple antibody assay and their coprecipitation with unlabelled mite allergens in CIE and CRIE. The latter studies showed that at least 6 of the 9 previously identified allergens from our laboratory [3], namely *Der p* I, Dpt 1, 3, 4, 8 and 17 were recognized. The failure to detect the remaining 3 allergens, namely Dpt 7, 22 and 31, in the lysates may be due to a variety of reasons such as destruction or loss of specific mRNA

during isolation, non-translatable mRNA or absence of or low levels of methionine in the primary sequence.

The labelled mite proteins were also analyzed by immunoprecipitation specifically to detect *Der p* I but we were unsuccessful. The reasons for this failure, in contrast to the apparent success in detecting it by CIE, are unclear but may reflect inherent differences in the affinities and or specificities of the different antisera used. The weak radiostaining obtained with *Der p* I in the CIE studies may reflect the low level of methionine (3 residues) contained therein [13]. The failure to detect coprecipitation with the non-translated control together with the reactivity of *Der p* I-specific oligonucleotide probes with clones from a mite cDNA library in *E. coli* [unpubl. observations] suggest that the CIE findings were valid.

In conclusion, data obtained in these studies indicated that it is possible to isolate biologically active mRNA from the house dust mite. Thus, the possibility of introducing mite cDNA into *E. coli* via a suitable expression vector with the concomitant synthesis of mite allergens is now very real and studies in our laboratory in this regard have now begun.

Acknowledgements

This study was supported by the Princess Margaret Children's Medical Research Foundation (PMCRF) and the National Health and Medical Research Council of Australia. We thank Ms B. Musulin and Ms A. Nisbet for excellent technical assistance and Ms C. Jones for typing the manuscript, Commonwealth Serum Laboratories for providing mites and the Audio Visual Production Unit, Sir Charles Gairdner Hospital, for photographic work. This is publication No. 260 of the PMCMRF.

References

- 1 Voorhorst, R.; Speikma-Boezeman, M.I.A.; Speikma, F.T.M.: Is a mite (*Dermatophagoides* sp.) the producer of the house dust allergen? *Allergie Asthma* 10: 329 (1964).
- 2 Maunsell, K.; Wraith, D.G.; Cunningham, A.M.: Mites and house dust allergy in bronchial asthma. *Lancet* i: 1267 (1968).
- 3 Stewart, G.A.; Butcher, A.; Lees, K.; Ackland, J.: Immunochemical and enzymatic analyses of *Dermatophagoides pteronyssinus*. *J. Allergy clin. Immunol.* 77: 14 (1986).
- 4 Lind, P.; Lowenstein, H.: Identification of allergens in *Dermatophagoides pteronyssinus* mite body extract by crossed radioimmunoelectrophoresis with two different rabbit antibody pools. *Scand. J. Immunol.* 17: 265 (1983).

- 5 Krilis, S.; Baldo, B.A.; Basten, A.: Antigens and allergens from the common house dust mite *Dermatophagoides pteronyssinus*. Part II. Identification of the major IgE binding antigens by crossed radioimmuno-electrophoresis. *J. Allergy clin. Immunol.* 74: 142 (1984).
- 6 Tada, T.: Regulation of reaginic antibody formation in animals; in Kallos, Walkman, de Weck, *Prog. Allergy*, vol. 19, p. 122 (Karger, Basel 1975).
- 7 Lanzavecchia, A.: Antigen specific interaction between T and B cells. *Nature* 314: 537 (1985).
- 8 Tovey, E.R.; Chapman, M.D.; Platts-Mills, T.A.E.: Mite faeces are a major source of house dust allergens. *Nature* 289: 592 (1981).
- 9 Aviv, H.; Leder, P.: Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. natn. Acad. Sci. USA* 69: 1408 (1972).
- 10 Stewart, G.A.: The isolation and characterization of the allergen Dpt 12 from *Dermatophagoides pteronyssinus* by chromatofocusing. *Int. Archs Allergy appl. Immun.* 69: 224 (1982).
- 11 Stewart, G.A.; Fisher, E.F.; Turner, K.J.: Antibodies to *Dermatophagoides pteronyssinus* allergen Dpt 12 contaminating rabbit antisera to human and mouse anti-immunoglobulins. *J. immunol. Methods* 61: 157 (1983).
- 12 Laemmli, U.K.: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680 (1970).
- 13 Stewart, G.A.; Simpson, R.J.; Thomas, W.R.; Turner, K.J.: Physicochemical characterization of a major protein allergen, *Der p I*, from the house dust mite *Dermatophagoides pteronyssinus*: amino acid analysis and circular dichroism studies. *Int. Archs Allergy appl. Immun.* (in press).

Received: October 28, 1986

Correspondence to: Dr. G.A. Stewart,
Clinical Immunology Research Unit,
Princess Margaret Hospital,
Thomas Street,
Subiaco 6008 (Western Australia)