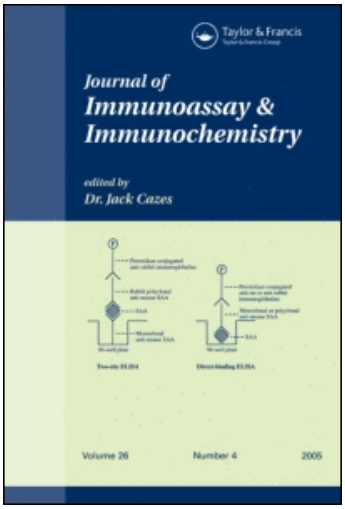

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A Microparticle Enhanced Nephelometric Immunoassay (Nephelia^{R*}) Applied to Thymulin Measurement

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A MICROPARTICLE ENHANCED NEPHELOMETRIC IMMUNOASSAY (NEPHELIA[R] *) APPLIED TO THYMULIN MEASUREMENT

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ABSTRACT

This article describes a microparticle enhanced nephelometric immunoassay (Nephelia[R]) applied to the quantification of the thymic peptide hormone thymulin. Nephelia[R] uses antibody recognition by the anti-thymulin antiserum in a competitive reaction between free thymulin and thymulin bound to the microspheres. The binding between microsphere and thymulin is achieved with the aid of a protein carrier. The sensitivity of the competitive reaction varied with the protein carrier and the antiserum sample. The most efficient reaction was obtained with the thymulin-metallothionein-microsphere conjugates; as little as 5 pg/ml of thymulin could be detected. In adult human serum, or in its ultra-filtrate, spiked synthetic thymulin was totally recovered. Measurement of thymulin in serum or ultra-filtrate samples demonstrated the presence of interference from molecules in the serum. Nephelia[R] led to the same conclusions as those reported with other methods and performs as well, and is more simple to use than radio- or enzymo-immunoassays for thymulin measurement.
(KEY WORDS : nephelometry; microparticle immunoassay; Nephelia[R]; synthetic thymulin; polyclonal antibodies.)

* NEPHELIA[R] is a registered trademark of DIAGNOSTICS PASTEUR, Marnes-la-Coquette, France

INTRODUCTION

Thymulin, discovered by Bach et al. (1), and formerly called "serum thymic factor", is a thymic hormone involved in several aspects of extra- and intra-thymic T cell differentiation (2). Thymulin is a nonapeptide (Glp-Ala-Lys-Ser-Glu-Gly-Gly-Ser-AsnOH) (3). The synthetic hormone has been shown to be biologically active (3) and demonstrates the same physicochemical characteristics as the natural hormone (4). Teams using immunochemical assays perfected for synthetic thymulin have reported interference from molecules in the serum (5-8). It is not possible to perform direct specific quantification of natural thymulin in serum samples; extraction of serum is still necessary to eliminate interfering biological molecules (8,9). In the absence of a direct biochemical assay, the determination of thymulin can still be done using a biological assay developed by Dardenne and Bach (10). Extensive use of the biological test is limited by the fact that it is only a semi-quantitative assay. Moreover other thymic hormones, such as thymopoietin (11) and thymosin α1 (12), as well as an allogeneic factor from activated T cells (13,14), also show activity in the biological assay. The determination of thymulin levels in biological fluids is critical for the study of immune disorders. Thus a specific method for direct measurement of thymulin is needed.

We used a new microparticle enhanced nephelometric immunoassay, Nephelia^R, which is simple, rapid and very sensitive, for thymulin measurement. Nephelia^R has very recently been successfully applied to serum and milk proteins (15-17) and is marketed by Diagnostics Pasteur (Marnes-la-Coquette, France). The synthesis of a stable microparticle

reagent on which polypeptides can be covalently coupled by simple incubation has been developed (18). The binding of several antigen molecules to one microsphere makes it possible to assay haptens such as thymulin. Immunonephelometry measures the light scattered by the turbidity of antigen-antibody complexes formed through the antigen-antibody reaction. The increased size of the antigen, due to its binding to the microsphere, and formation of an enlarged antigen-antibody complex, provides for earlier detection of the reaction by the nephelometer, giving improved accuracy to this method. We describe the application of Nephelia^{R} to thymulin measurement in a competitive reaction between free thymulin and the microsphere-bound peptide, for binding to polyclonal antibodies.

MATERIALS AND METHODS

Reagents

Synthetic thymulin (S8256), chicken ovalbumin (A5503), polyaspartic acid (PAA) (P5387), and metallothionein (MT) (M7641) were obtained from Sigma (St Louis, MO). N-(3 dimethylaminopropyl)-N ethyl-carbodiimide hydrochloride, acrolein (800178), methacrylic acid (800578), hydroxyethylmethacrylate (800588) were provided by Merck (Darmstadt, F.R.G.). N,N'-methylene bisacrylamide was obtained from Eastman Kodak Company (Kingsport, Tennessee). Bovine serum albumin (BSA) was from IBF (Villeneuve la Garenne, France). All other chemical reagents were of analytical reagent grade. Freund's complete or incomplete adjuvant was from Behring Institut (Marburg, F.R.G.). Polystyrene micro-cuvettes were from Ratiolab (Buchschlag, F.R.G.).

Anti Thymulin Antiserum

Synthetic thymulin was covalently bound to chicken ovalbumin carrier (1/4, w/w) in the presence of 4 mM glutaraldehyde (19). The mixture was stirred for 3 hours at room temperature. The reaction was stopped using 15.8 mM sodium bisulfite and the conjugate dialyzed against 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl. Eight six-week-old "Roux de Bourgogne" rabbits were immunized according to a protocol previously described by Monier et al. (20) and already used by our team (21). For each shot, immunogen containing 0.5 mg thymulin was injected. Rabbits received multiple subcutaneous injections with ovalbumin-thymulin conjugate in the presence of Freund's complete adjuvant (1/2, v/v). Four months later, the rabbits were boosted with immunogen and incomplete Freund's adjuvant (1/2, v/v). The booster dose was then again administered after 10 weeks, and yet again 4 months later.

Synthesis of the Microparticle Reagent

The microparticle manufacturing process is covered by a patent (18). Microspheres employed here were obtained by irradiation, under a cobalt 60 source (ORIS, Nucleart, CENG, France) (23 Krad/cm2/h for 3 hours), of a mixture containing 5 % (v/v) total monomers (of the 5 % : 49.7 % (v/v) hydroxyethylmethacrylate, 47 % (v/v) acrolein, 2 % (v/v) methacrylic acid and 1.3 % (w/v) N,N'-methylene-bis-acrylamide) in a 0.6 g/l sodium dodecyl sulfate solution. Microspheres thus obtained had an average diameter of 105 nm (measured by transmission electron microscopy). Microspheres were hydrophilic, stable in suspension and reactive, which allowed covalent coupling with the protein. Microspheres were stored at +4°C in a reducing medium (hydroquinone).

Preparation of the Protein-Microsphere Conjugates

Three proteins were used : bovine serum albumin (BSA), polyaspartic acid (PAA) and metallothionein (MT). Coupling medium was 0.3 M NaCl, 0.1 M borate buffer, pH 8.2 for BSA, and 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2 for the other two carriers. Coupling was carried out by an overnight incubation (at +4°C) of the protein with 10 mg/ml microspheres previously dialyzed in the coupling medium. Microsphere aldehyde groups which had not reacted were blocked with 0.12 M 2-aminoethanol, prepared in coupling medium buffered with acetic acid at pH 8, for 1 hour at room temperature. Microspheres grafted with PAA were saturated with final 0.036 M glycin instead of 2-aminoethanol. Imine bonds between microsphere and PAA were reduced with sodium borohydride (BH_4Na) (5 mg for 10 mg microspheres) at pH 8-9 during 2 hours stirring. The pH was then adjusted to 6 with 1 M acetic acid. After coupling, excess BSA or PAA was eliminated by ultracentrifugation on a 200-800 g/l discontinuous sucrose gradient then dialyzed in coupling medium, while MT, whose molecular weight is 6 400 D, was simply dialyzed. 10 mg of 105 nm diameter microspheres were coupled with either 2×10^{-8} BSA moles, 5×10^{-7} PAA moles or 2×10^{-8} MT moles.

Coupling of Thymulin to the Protein-Microsphere Conjugates

Carboxylic groups of the bound protein were activated in 0.14 M NaCl, pH 6.5, by N-(3 dimethylaminopropyl)-N ethyl-carbodiimide hydrochloride (22), 5 mg per 10 mg of protein-microspheres. Thymulin was then added, and the whole preparation incubated for 4 hours at room temperature. 4 M urea (250µl/10 mg microspheres) was added to block the remaining activated groups of the proteins. Thymulin-protein microspheres

thus prepared were stored at +4°C in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2 after dialysis to eliminate excess urea and uncoupled thymulin. 10 mg of BSA-microspheres, PAA-microspheres and MT-microspheres were coupled with 10^{-6}, 10^{-8} and 10^{-7} thymulin moles respectively.

Assay Buffers

The assay used 0.05 M sodium phosphate buffer, pH 7.2, containing 0.33 M NaCl, 2g/l Triton X100 and 2 g/l NaN_3. Development of the assay medium was carried out by choosing buffer components according to the results of the agglutination reaction. In some experiments, the assay was performed with the addition of zinc salt. In this case the non sequestrating assay buffer was 0.01 M HEPES, pH 7.5, containing 0.2 M NaCl, 2 g/l Triton X100, 2 g/l NaN_3 and the additives chosen for phosphate buffer.

Assay Procedure

All reagents were prepared in the assay buffer. All reactions were performed at room temperature and the result was measured using a Behring nephelometer (Behring Institut, Marburg, F.R.G.). The nephelometric reference signal was given by the agglutination of thymulin-protein microspheres with anti-thymulin antibodies. The reaction was performed in a total volume of 300 µl; antiserum (150 µl) was prepared in a serial dilution in each cuvette then 150 µl of the thymulin-protein microspheres were added. The agglutination curve was drawn and used to choose efficient concentrations of thymulin-protein microspheres and dilution of antiserum. For the competitive reaction, synthetic thymulin (150 µl) was first added in a serial dilution, antiserum (75 µl) was then added. Both

were incubated together for 30 min before thymulin-protein microspheres (75 µl) were added. For the assay of biological samples, 150 µl of the sample diluted in 0.14 M NaCl, pH 6.5, or 150 µl of only 0.14 M NaCl, pH 6.5 for the reference signal, were first added, antiserum and the thymulin-protein microspheres were then added as described above but diluted in a 2 times concentrated assay buffer. The results are shown as intensity of scattered light, i.e. voltage recorded by the nephelometer, in relation to thymulin concentration. Agglutination percentage compared with the nephelometric reference signal can thereby be calculated.

Serum Samples

Serum samples were obtained from 50 children and 92 adult donors. The serum was ultra-filtered by centrifugation, at room temperature, using ready for use Centrisart-I tubes (Sartorius, Göttingen, F.R.G.) containing a filtration membrane with a cut-off at the molecular weight of 20 000 D. A preliminary verification showed that thymulin-protein microspheres were stable at usual working dilutions in human serum and ultra-filtrate samples.

For recovery studies, synthetic thymulin was added to a pool of sera from 15 adult donors, supposedly without native thymulin, and recovery measured in the 1/300 serum and 1/4 ultra-filtrate. At the same time, a standard curve was established in each of the two biological media (1/300 serum and 1/4 ultra-filtrate) and was directly used to express thymulin concentration results.

Precision of the recovery was assessed by measuring 3 concentrations of thymulin in 1/4 ultra-filtrate 20 times with the same standard curve on the same day (within-run precision), and over 9 days by 5 assays repeated on

days 1, 2, 5, 7, and 9 with a new standard curve each day (between-run precision).

RESULTS

Antisera

Normal rabbit serum obtained before immunization showed no agglutination with any of the three thymulin-protein microspheres. No agglutinating anti-thymulin antibodies were obtained after the first injection, and after the first booster injection, antibodies were produced after two weeks. Three different antiserum samples, taken from the same rabbit, were used for all the reactions presented here. They were A antiserum (32 days after the first booster injection), B antiserum (42 days after the first booster injection), C antiserum (12 days after the second booster injection).

Influence of the Reaction Medium on Agglutination

Compared to the complete buffer tested (0.05 M sodium phosphate, pH 7.2, containing 0.33 M NaCl, 30 g/l PEG 6000, 1.50 mM EDTA, 2 g/l Triton X100 and 2 g/l NaN_3), EDTA-free buffer did not modify the agglutination intensity (Fig. 1), which is understandable because phosphate buffer is also a chelating medium for divalent ions. It is essentially the PEG which was responsible for the amplification of the agglutination reaction. The pH sensitivity of the agglutination reaction was assessed under the same working conditions as in Fig. 1. The agglutination reaction was performed in the complete phosphate buffer at pH 7.5 and 8.0 in comparison with pH 7.2 initially chosen. Maximum agglutination was obtained at 1/96 antiserum for

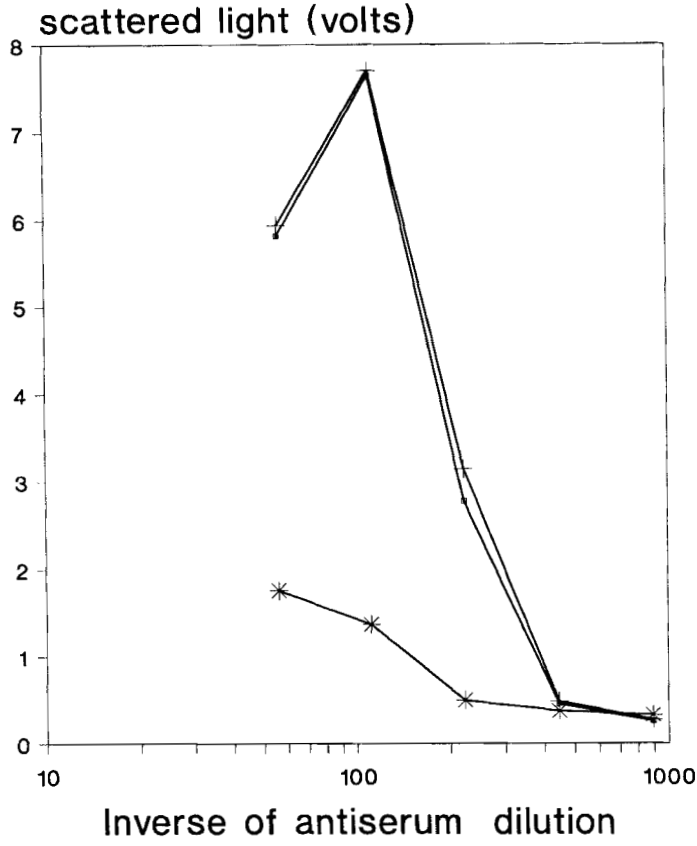

FIGURE 1. Agglutination of 0.2 mg/ml thymulin-MT-microspheres by B antiserum in different buffers : complete buffer (0.05 M sodium phosphate, pH 7.2, containing 0.33 M NaCl, 30 g/l PEG 6000, 1.50 mM EDTA, 2 g/l Triton X100 and 2 g/l NaN_3) (⊡), complete buffer without EDTA (+), and complete buffer without PEG (*), t = 120 mn.

all pH values. The signals were 6.92, 6.08 and 2.40 volts for pH 7.2, 7.5 and 8.0 respectively. Results therefore differed little at pH 7.5 but were much lower at pH 8.0.

To study a medium allowing the addition of zinc, complete HEPES buffer was used, with EDTA, or without EDTA with added zinc chloride

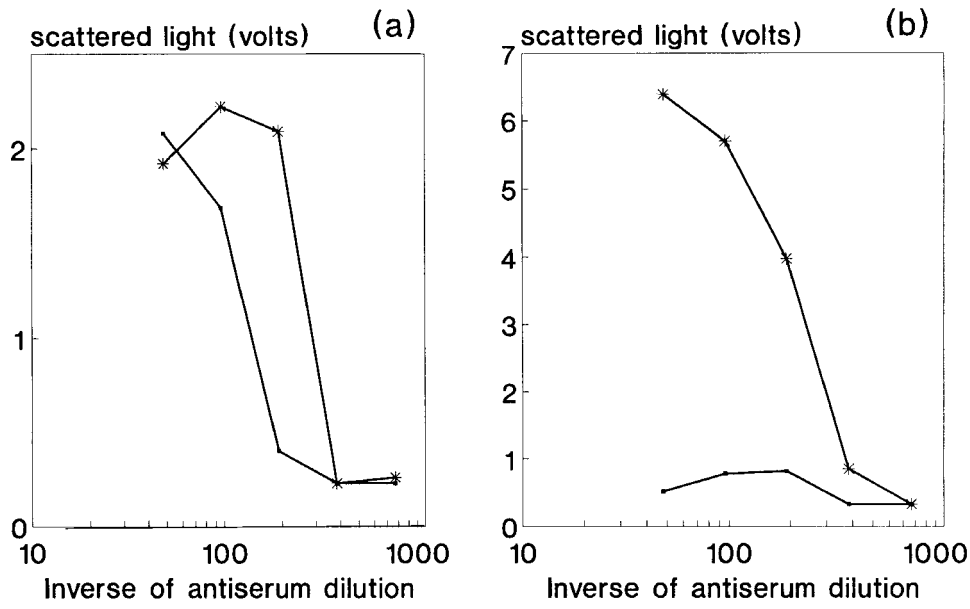

FIGURE 2. Agglutination reaction with the C antiserum in 0.01 M HEPES, pH 7.5 (containing 0.2 M NaCl, 30 g/l PEG 6000, 2 g/l Triton X100 and 2 g/l NaN_3) supplemented with 1.50 mM EDTA (*) or with 0.56 μM zinc (◻), t=120 mn, of (a): 0.05 mg/ml thymulin-PAA-microspheres and (b): 0.05 mg/ml thymulin-MT-microspheres.

($ZnCl_2$) resulting in a zinc concentration of 0.56 μM. The agglutination of the thymulin-PAA-microspheres (Fig. 2-a) was weak but most intense in the presence of EDTA. This difference increased when thymulin-MT-microspheres were used (Fig. 2-b). MT was therefore the protein carrier with the most sensitive agglutination reaction to the presence of zinc or EDTA in the medium.

All these results showed the sensitivity of the agglutination reaction to changes in the reaction medium. 0.05 M sodium phosphate, pH 7.2, containing 0.33 M NaCl, 30 g/l PEG 6000, 1.50 mM EDTA, 2 g/l Triton

TABLE 1

Competitive reaction of synthetic thymulin and the 3 kinds of thymulin-protein-microspheres with the A antiserum, in complete phosphate buffer.

Spacer	micro-spheres mg/ml	A Antiserum Dilution	Assay Time (min)	Concentration of Thymulin (ng/ml) required to give % of agglutination of		
				90 %	50 %	10 %
BSA	0.083	1/100	105	0.214	21.63	80.98
PAA	0.200	1/200	100	0.354	8.78	39.13
MT	0.130	1/75	60	0.078	1.11	15.18

X100 and 2 g/l NaN_3, called complete buffer, gave the best results and was consequently chosen for further tests.

Optimisation of Working Conditions : Choice of Protein Carrier and Antiserum Batch for the Competitive Reaction

Assessment of the optimal assay time, the optimal concentration of the three kinds of thymulin-protein microspheres and the optimal dilution of the A antiserum for the competitive reaction led to the use of different conditions in each case to obtain the lowest concentration of competing thymulin required to give a significant signal reduction (Table 1). Agglutination was entirely specific since synthetic thymulin was able to totally inhibit the reaction. The most sensitive reaction was obtained with the thymulin-MT-microspheres, whereas the least sensitive reaction was obtained with the thymulin-BSA-microspheres. Fig. 3 shows the competitive

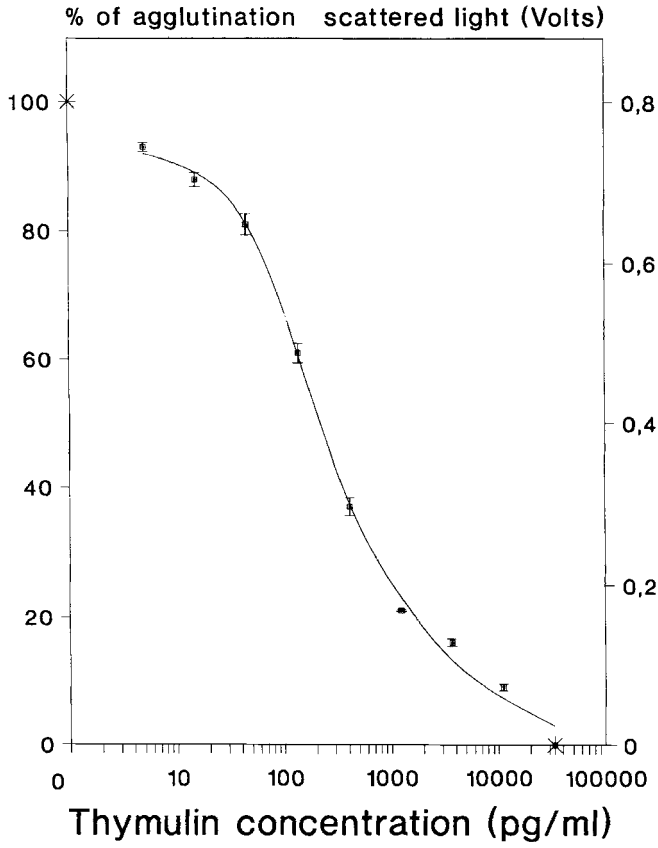

FIGURE 3. Competitive reaction of synthetic thymulin and 0.033 mg/ml thymulin-MT-microspheres with 1/400 C antiserum, in complete phosphate buffer, t = 180 mn (standard curve). Error bars for triplicate measurements are presented.

reaction between thymulin-MT-microspheres and synthetic thymulin, with the C antiserum. A thymulin concentration of 226 pg/ml inhibited 50 % of the reference agglutination reaction and was a lower concentration than that required for the same inhibition percentage with the A antiserum (shown in Table 1). Detection was consequently better when using the C antiserum.

These results were used to construct the standard curve. Reference agglutination was decreased by 10 % with 9.7 pg/ml thymulin and the thymulin concentration of 5 pg/ml led to 93 % of reference signal.

Assay in Serum and Ultra-Filtrate Samples

Comparison between concentration of added and recovered thymulin in biological media is given in Fig. 4. The synthetic thymulin was correctly recovered between 8 and 1000 pg/ml in 1/4 ultrafiltrate ($r=0.9962$, mean recovery percentage was 100.18 ± 14.30), and between 16 and 500 pg/ml in 1/300 serum ($r=0.9983$, mean recovery percentage was 102.56 ± 23.16) (Fig. 4).

Within- and between-run CV studied for 3 concentrations of synthetic thymulin ranged between 5.5 and 12.5 % (Table 2). Stability of thymulin can be shown by the variations between day 1 and day 9 which were : from 7.4 to 4.9 pg/ml, from 90.8 to 73.1 pg/ml and from 1061.5 to 881.2 pg/ml for the 3 concentrations studied.

Samples of serum and ultra-filtrate were assayed in the competitive reaction. The results were expressed in thymulin concentration according to the standard curve. The test of serial dilutions by 2 of the serum pool of 15 adult donors showed that the concentration of thymulin was linear between 1/80 and 1/2560 ($y=1.39x-44.35$; $r=0.9906$). Mean thymulin concentrations measured in 4 serum dilutions showed no difference between sera of 7 adult donors, supposedly without thymulin, and sera of 14 children, which should contain thymulin (Table 3). When taking into account the serum 1/40 dilution, serum thymulin levels do not dilute in parallel with the standard preparation. The test of serial dilutions by 2 of the ultra-filtrate samples

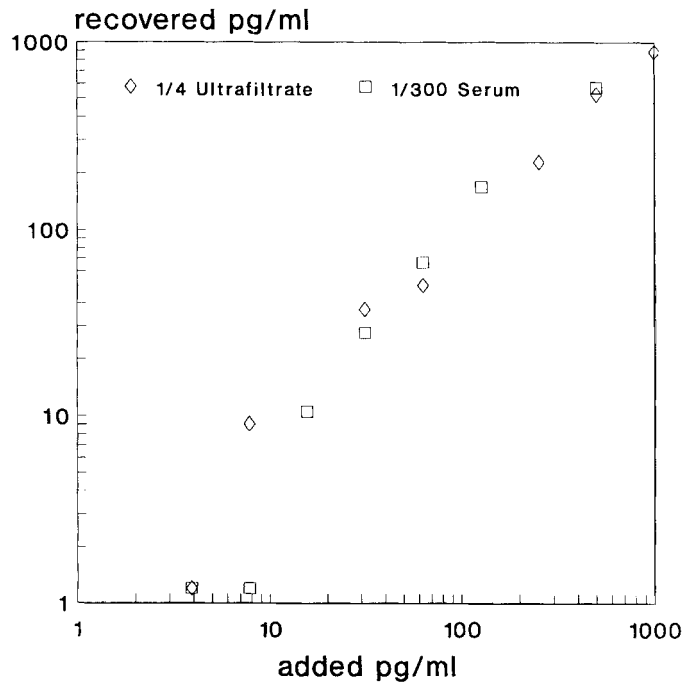

FIGURE 4. Synthetic thymulin concentration (pg/ml) recovered after incorporation into a serum pool from 15 adult donors then addition in the Nephelia^{R} assay into 1/300 serum pool or into 1/4 serum pool ultra-filtrate, according to a standard curve established in each of the two biological media (1/300 serum and 1/4 ultra-filtrate).

showed a thymulin concentration of 31 ± 8 pg/ml in 1/2 ultra-filtrates of 12 children and 111 ± 12 pg/ml in 1/2 ultra-filtrates of 6 adult donors. Concentrations in more diluted ultra-filtrates were lower than expected (data not shown). Ultra-filtrate samples were assayed at the 1/2 dilution because they inhibited the reference agglutination far less than the whole serum. The division of the ultra-filtrate samples into different thymulin concentration groups (Fig. 5) showed that thymulin concentration was higher in ultra-filtrates of serum from 70 adult donors than in those from 36

TABLE 2

Within- and between- run precisions of Nephelia^{R} for synthetic thymulin recovered in 1/4 ultra-filtrate. SD = standard deviation ; CV = coefficient of variation.

Within - run				Between - run			
n	Mean(SD),pg/ml		CV,%	n	Mean(SD),pg/ml		CV,%
20	4.2	(0.3)	7.1	5	6.4	(0.8)	12.5
20	67.0	(3.7)	5.5	5	78.9	(7.1)	8.9
20	723.8	(48.0)	6.6	5	945.1	(82.6)	8.7

TABLE 3

Mean (± SEM) thymulin concentration (pg/ml) measured in 4 dilutions of serum from adult donors and children according to the standard curve. There was no significant difference between the groups at any dilution ($p>0.05$) (statistical analysis was performed by Student's t test).

	serum dilution			
	1/40	1/160	1/640	1/2560
children n=14	1184 (126)	679 (106)	117 (27)	34 (12)
adults n=7	1125 (168)	639 (154)	111 (35)	40 (5)

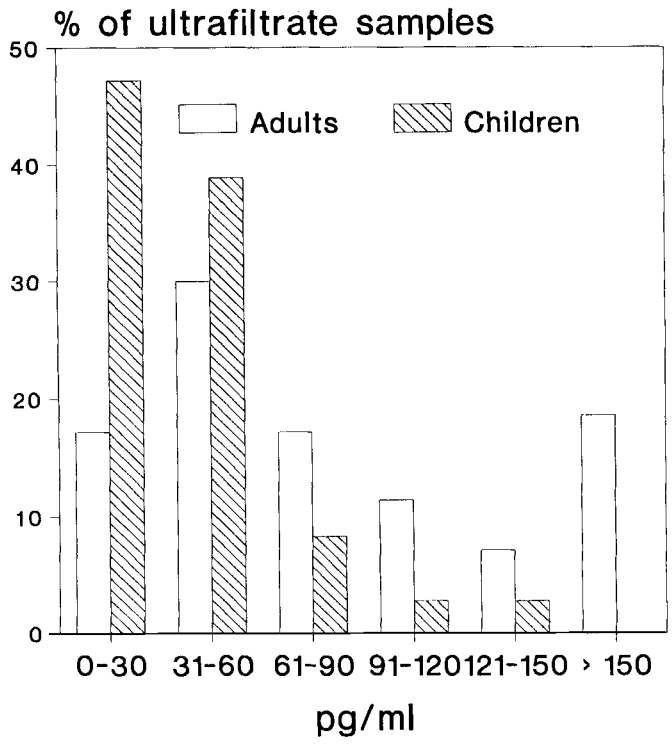

FIGURE 5. Division into thymulin concentration groups of 1/2 ultrafiltrates of sera from 36 children and 70 adult donors, according to the standard curve.

children. 47 % of adult donors samples showed a thymulin concentration between 0 and 60 pg/ml, while 86 % of children samples showed thymulin concentration in this range. No result above 150 pg/ml was found in children samples, whereas 18 % of adult donors samples showed a thymulin concentration above 150 pg/ml (maximum value was 1 606 pg/ml).

DISCUSSION

In the present report we describe a potentially useful and sensitive immunochemical approach to the measurement of thymulin by a microparticle enhanced nephelometric immunoassay (Nephelia^R). The assay is a competitive immunological reaction between the synthetic hormone bound to the microsphere and free thymulin, using a specific antiserum.

The coupling of thymulin required the microspheres to be first coated with a protein carrier because the hormone is too small a molecule to provide, on its own, an adequate covering, which is essential for the stability and the immunoreactivity of the microsphere. On the microsphere and in the immunogen, thymulin was attached to the protein carriers principally through the ε-amino group of Lys-3. The chemical amide link, created by carbodiimide on the microsphere protein carrier, was therefore different from the one created with glutaraldehyde in the immunogen. This avoided any immunological cross-reaction with the coupled microspheres through linkage, which should lead to a non-thymulin specific agglutination. The antigenicity of thymulin was maintained in both cases of coupling since the C-terminal residue was the same for antibody recognition. Indeed, studies on structure-activity relationship with varied synthetic analogs have shown that neither Glu_1 or Ala_2 (9) nor the side chain of Lys-3 (23) were necessary for antigenicity. On the contrary, with regards to the C-terminal residue, all modifications would abrogate antibody recognition (24).

There are two forms of thymulin, one biologically active containing zinc, and the other inactive, free of the metal (25). The presence of this metal also makes it possible for a new monoclonal antibody-defined epitope

to appear (26). We chose a zinc chelating medium to assay the whole peptide regardless of the varied conformations due to the binding of zinc on thymulin. On the microsphere, the accessibility of thymulin depends on the protein carrier. Thymulin-MT-microspheres agglutinated by the best antiserum (C) comprised the most sensitive detection of synthetic thymulin : 5 pg/ml at 93 % reference agglutination. Thus Nephelia^{R} is sufficiently sensitive to be used for quantitating the levels of thymulin in human serum.

For the recovery test, synthetic thymulin was largely distributed over the concentration range of the standard curve. Synthetic thymulin spiked into a serum pool of adult donors was almost completely recovered in a dilution of serum or ultra-filtrate. The assay of free synthetic thymulin was possible in the whole serum or after the ultra-filtration procedure, and we tried to apply Nephelia^{R} for the direct measurement of thymulin content in serum and ultra-filtrate samples. The high thymulin concentrations measured both in children or adult donors serum samples, supposedly with and without thymulin respectively, and the higher thymulin concentration measured in ultra-filtrates of adult donor sera when compared with ultra-filtrates of children sera, were not consistent with what could be expected for the natural thymulin alone. Moreover, a comparison of the results obtained from the assay of thymulin in serum and ultra-filtrate samples showed that values were approximately 200 times higher in serum than in ultra-filtered serum, after correcting for dilution. We concluded that there are molecules in serum, partly recovered in the ultra-filtrate, that interfere with the reaction.

Molecules in the serum interfering with the biological assay of thymulin have been reported by Bach et al. (2) as having high or low

molecular weight (between 100 000 and 300 000 D and also between 4 000 and 20 000 D); their level seemed not to vary with age or with blood thymulin level. A high molecular weight molecule still interfered with the biological assay when serum was of low concentration (1/5 000) (1). Interfering molecules in the serum have also been reported by teams who used immunochemical assays perfected for synthetic thymulin (5-8). It is not possible to perform a specific quantification on biological samples directly. Serum extraction is still necessary to eliminate biological molecules which interfer, and the low concentration of thymulin in blood requires sample concentration before assay (8,9). Attempts to measure natural serum hormone with radio-immunoassays have given values, in the pig, of 100-500 pg/ml (5) or 32 pg/ml (2) or 22 pg/ml (9) and, in the mouse, of 45 pg/ml (2) or 1.3 pg/ml (9). A level of 44 pg/ml has been measured in children (6) and of 1.49 pg/ml in young people (8). Contradictions in these results reflect the considerable complexity of assaying natural thymulin.

Nephelia^R led to the same conclusions as those reported with other methods already published, and performs as well. Nephelia^R, giving rapid results after only a single-step reaction using three reagents without a washing or separation phase, is more simple to use than radio- or enzymo-immunoassays. Nephelia^R is therefore useful in further fundamental work to better characterize interfering molecules reported in all biological or immunochemical assays of thymulin. Such results are required for the development of accurate immunochemical assays for natural thymulin. We can hope that Nephelia^R could then be useful for direct measurement of thymulin in biological media. Moreover, in addition to several assays with Nephelia^R already published (15-17), our work describes the first

application of this method for the measurement of an hapten and points the way to a possible application of Nephelia^{R} in this type of assay.

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