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A MICROPARTICLE ENHANCED NEPHELOMETRIC

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

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.

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

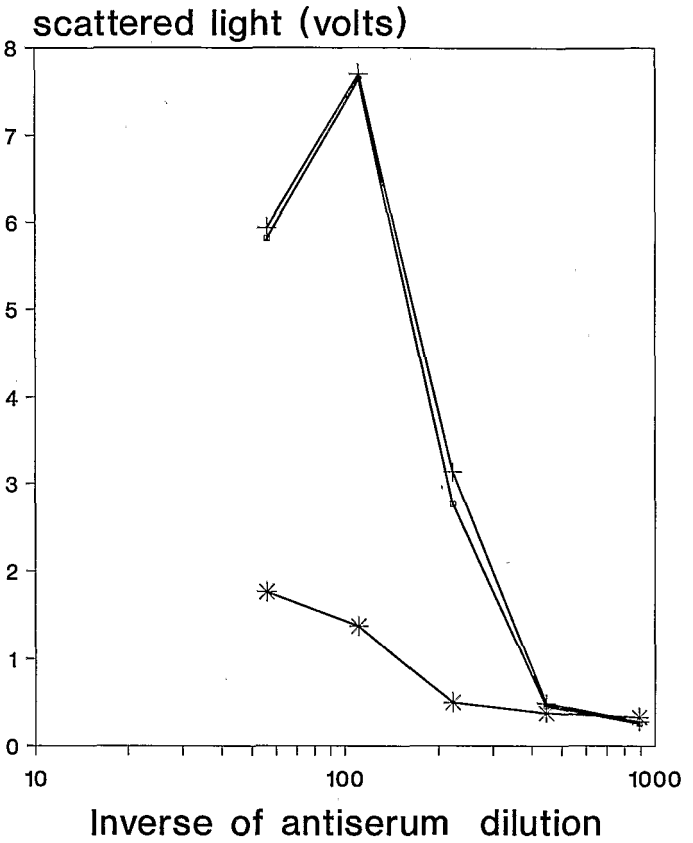

FIGURE 1. Agglutination of 0.2 mg/ml thymulin-MT-microspheres by B antiserum in different buffers : complete buffer (0.05 M sodium phosphate, pH 7.2, containing 0.33 M NaCl, 30 g/l PEG 6000, 1.50 mM EDTA, 2 g/l Triton X100 and 2 g/l NaN_3) (▫), complete buffer without EDTA (+), and complete buffer without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

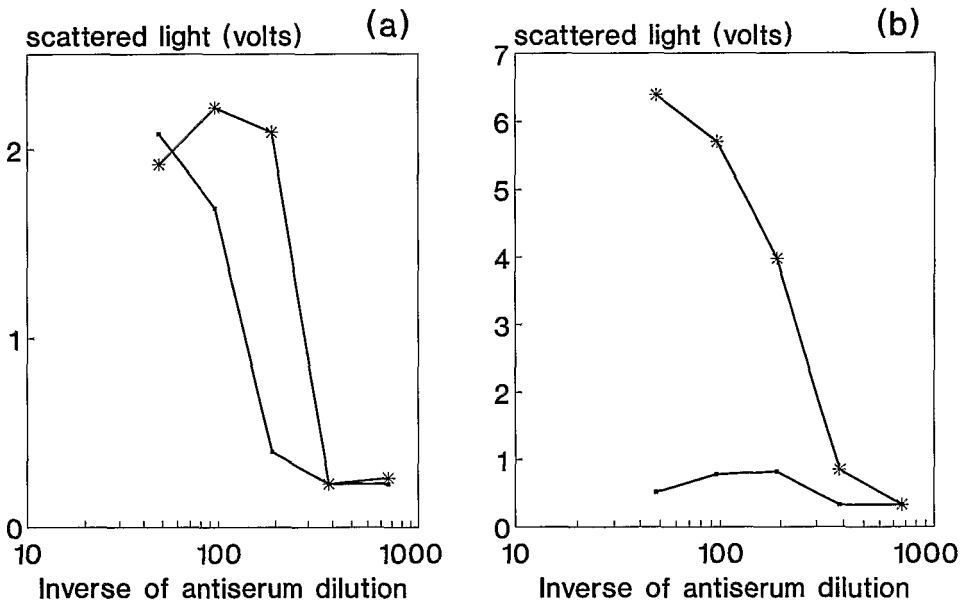

FIGURE 2. Agglutination reaction with the C antiserum in 0.01 M HEPES, pH 7.5 (containing 0.2 M NaCl, 30 g/l PEG 6000, 2 g/l Triton X100 and 2 g/l NaN_3) supplemented with 1.50 mM EDTA (*) or with 0.56 μM zinc (▫), t=120 mn, of (a): 0.05 mg/ml thymulin-PAA-microspheres and (b): 0.05 mg/ml thymulin-MT-microspheres.

($ZnCl_2$) 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

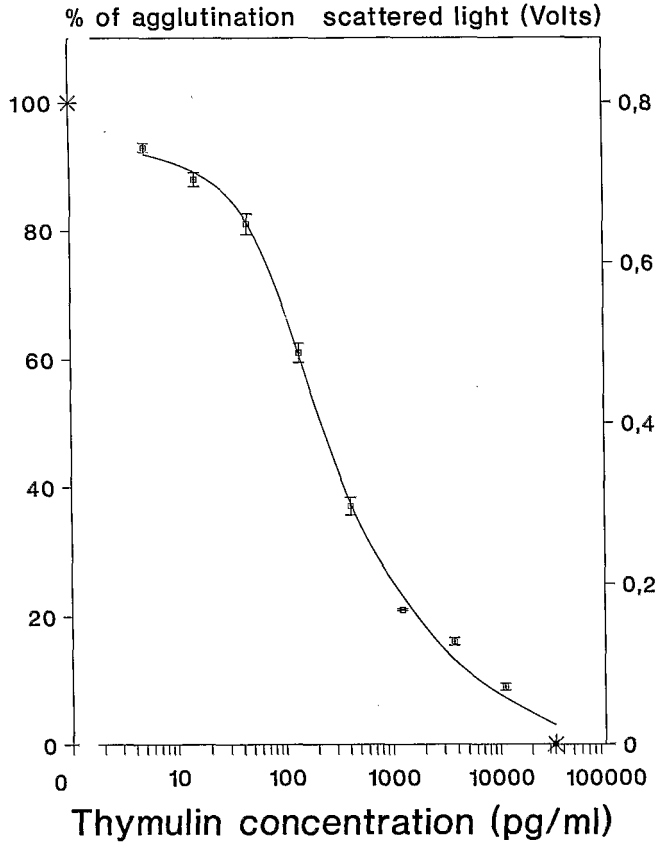

FIGURE 3. Competitive reaction of synthetic thymulin and 0.033 mg/ml thymulin-MT-microspheres with 1/400 C antiserum, in complete phosphate buffer, t=180 mn (standard curve). Error bars for triplicate measurements are presented.

reaction between thymulin-MT-microspheres and synthetic thymulin, with the C antiserum. A thymulin concentration of 226 pg/ml inhibited 50 % of

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

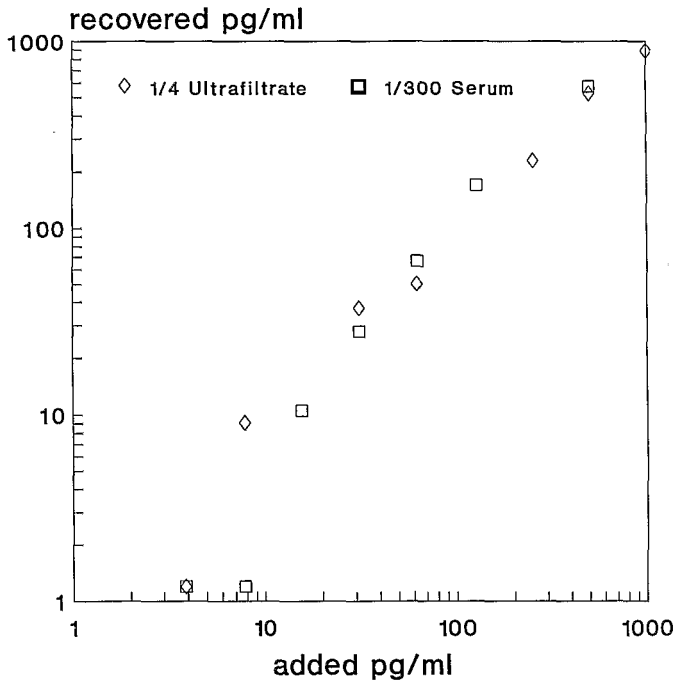

FIGURE 4. Synthetic thymulin concentration (pg/ml) recovered after incorporation into a serum pool from 15 adult donors then addition in the Nephelia^{R} assay into 1/300 serum pool or into 1/4 serum pool ultra-filtrate, according to a standard curve established in each of the two biological media (1/300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

[redacted]

by [redacted] Student's t test).

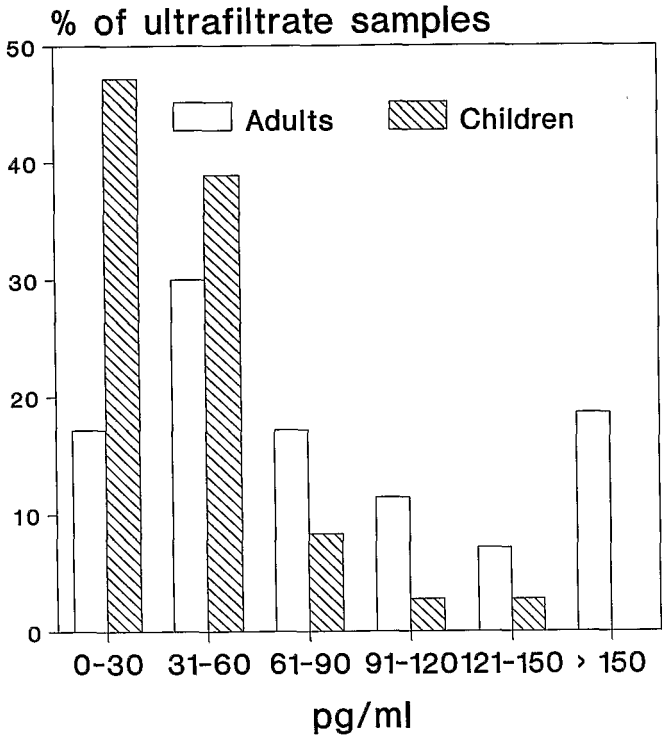

FIGURE 5. Division into thymulin concentration groups of 1/2 ultrafiltrates of sera from 36 children and 70 adult donors, according to the standard curve.

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

DISCUSSION

In the present report we describe a potentially useful and sensitive

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

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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