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Immunology

A zinc-dependent epitope on the molecule of thymulin, a thymic hormone

(thymus/monoclonal antibodies/rosette assay/immunofluorescence)

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ABSTRACT **Thymulin is a nonapeptide hormone produced by thymic epithelial cells. Its biological activity is strictly dependent on the presence of the metal zinc in the molecule. Antithymulin monoclonal antibodies have been produced against either the synthetic (AS_1) or the natural intraepithelial (AE_1) molecule. These monoclonal antibodies were screened for their abilities to inhibit the zinc-dependent biological activity of the hormone and were shown to bind to thymic epithelial cells. By using biological and immunofluorescence assays, the two antibodies were shown to recognize exclusively the zinc-coupled thymulin molecule. Other antithymulin antibodies screened by RIA or ELISA (using a zinc-deprived substrate) recognized a zinc-independent epitope on the thymulin molecule. These data indicate the existence of a zinc-specific conformation on the thymulin molecule. They are in agreement with NMR studies showing that the zinc-containing hormone has a unique structure.**

Thymulin is a thymic hormone known to induce intra- and extrathymic T-cell differentiation (1). It is a nonapeptide with the following amino acid sequence: <Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH (2). Its exclusive presence in the thymus was initially suggested by its strict thymus dependency and, more recently, by its unique immunohistochemical detection in thymic epithelial cells (TEC) (3–5). We showed previously that thymulin requires the presence of zinc to express its biological activity (6). The natural peptide completely loses its biological activity (as measured by a rosette inhibition assay) after being incubated with the chelating agent Chelex 100 and is reactivated after treatment with $ZnCl_2$ and, to a lesser extent, other metals—particularly, aluminium and copper. Although it has not yet been established definitively, much evidence suggests that thymulin incorporates zinc before being secreted by TEC (6, 7).

The interaction between zinc and thymulin has been demonstrated directly by the chromatography of a mixture of Chelex 100-treated [^{3}H]thymulin and $^{65}Zn^{2+}$ on Bio-Gel P-2. [^{3}H]Thymulin and bound ^{65}Zn coeluted precisely with the peak of thymulin biological activity (6) and the binding affinity was calculated to be around 1 μM by equilibrium chromatography (8). More recently, by means of NMR, it has been shown that the conformation of the metal-deprived peptide is strikingly different from that of the zinc–peptide complex.‡

All of these results indicate that natural thymulin binds zinc *in vivo* (probably within the thymus) and that this binding induces conformational changes necessary for the expression of biological activity. We report here that these changes are also associated with the alteration of antigenicity as assessed by use of antithymulin monoclonal antibodies.

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MATERIALS AND METHODS

Antithymulin Antibodies. The monoclonal antibodies AS_1 and AE_1 were raised in BALB/c mice after immunization with synthetic thymulin coupled to bovine serum albumin (9), or cultured human TEC (10), respectively. Supernatant cultures were selected by their capacities to inhibit thymulin activity *in vivo* and *in vitro* and by their selective positive reactivities in an indirect immunofluorescence (IF) assay on thymic cultures and frozen sections. The antisynthetic thymulin monoclonal antibody (AS_1) used was an IgG1, whereas the antiepithelial thymulin monoclonal antibody (AE_1) was an IgG2b. Both antibodies were used at the concentration of 0.1 mg/ml. The third monoclonal antibody (MA-FTS), kindly donated by K. Ohga, was produced by immunizing mice with thymulin coupled to mouse IgG and selected by an ELISA (11). Although this monoclonal antibody absorbs the thymus activity from human serum, it does not bind, in IF, to TEC (unpublished observations). A polyclonal antiserum was obtained by immunizing rabbits with thymulin coupled to bovine serum albumin as described (12). Antithymulin antibodies were purified by using affinity chromatography on a CN-Sepharose 4B column and were characterized by their capacities to bind ^{125}I-labeled thymulin (^{125}I-thymulin) in a RIA and to recognize synthetic as well as natural thymulin (12). The general characteristics of the various antithymulin antibodies are summarized on Table 1.

Other Reagents. The goat anti-IgG1 fractions of mouse immunoglobulins bound to fluorescein isothiocyanate (GAM/IgG1/FITC) and GAM/IgG2b/FITC were purchased from Nordic (Tilburg, Netherlands) and absorbed with rat organ powder prior to use. They were used at a dilution of 1:20. Thymulin (FTS-Zn) was synthesized by P. Lefrancier (Inst. Choay, Paris) according to an unpublished protocol.

Metal salts ($FeSO_4$, $ZnCl_2$, $CuSO_4$, $Al_2Si_2O_7$) of the highest reagent grade were purchased from Pasteur Institute, Paris. The metal-chelating agent Chelex 100 was produced by Bio-Rad (Paris).

IF Technique. Unfixed frozen sections (2 μm thick) of human thymuses or methanol-fixed epithelial cell cultures from human thymic explants were processed as described (13). The AS_1 monoclonal antibody was revealed by using GAM/IgG1/FITC, whereas AE_1 was revealed by using GAM/IgG2b/FITC.

Chelation and Metal Treatment. Human or mouse serum (100 μl) and synthetic thymulin (10 μg) were incubated for 30 min at room temperature with an equal volume of 5% Chelex 100. The mixture was then centrifuged at 1200 × *g* for 2 min to eliminate the chelating resin, and the biological activity of the supernatant was measured. In some experiments, 10 ng

Abbreviations: TEC, thymic epithelial cell(s); IF, immunofluorescence; GAM, goat anti-mouse; FITC, fluorescein isothiocyanate.

‡Laussac, J. P., Pasdeloup, M., Haran, R., Lefrancier, P., Choay, J., Dardenne, M. & Bach, J. F., Twelfth Conference on Magnetic Resonance in Biological Systems, 1984, Goa, India, p. 135.

Table 1. Characteristics of antithymulin antibodies

Antibody*	Immunogen	Screening assay
Monoclonal		
AS_1 (10)	Synthetic thymulin-BSA	Rosette inhibition + IF
AE_1 (11)	Human TEC	Rosette inhibition + IF
MA-FTS (12)	Synthetic thymulin-IgG	ELISA† + IF
Polyclonal		
antiserum (13)	Synthetic thymulin-BSA	RIA‡

BSA, bovine serum albumin.
*References cited in parentheses.
†Microplates coated with glutaraldehyde/thymulin.
‡Microplates coated with ^{125}I-thymulin as the radiolabeled tracer.

or 100 ng of $ZnCl_2$, $FeSO_4$, or $Al_2Si_2O_7$ was added to 100 μl of Chelex 100-treated serum or 1 μg of Chelex 100-treated thymulin. The mixture was incubated for 15 min at room temperature and its biological activity was measured in the rosette assay described elsewhere (6).

Absorption of Thymulin Biological Activity by Antithymulin Monoclonal Antibodies. The capacities of the various antithymulin antibodies to bind to thymulin were evaluated by their abilities to abrogate thymulin's biological activity in absorption experiments. The antibodies (100 μl) were preincubated for 30 min at 37°C with 100 ng of synthetic thymulin. The mixture was filtered through Amicon MPS_1 membranes and the residual biological activity of the filtrate was assessed by the rosette assay. The incubations were performed by using either the Chelex 100-treated peptide or the peptide coupled to zinc, iron, aluminium, or copper salts. Similar experiments were performed by using natural thymulin present in normal mouse or human serum. We also used serum from children suffering from nephrotic syndrome with zinc deficiency, in which we observed decreased thymulin biological activity that could be restored *in vitro* by the addition of $ZnCl_2$ (14), and serum from zinc-deficient mice, which also presented decreased thymulin activities restorable by the *in vitro* addition of $ZnCl_2$ (15). Finally, absorption experiments were performed with supernatants from human TEC cultures.

Thymulin Capacity to Inhibit Antithymulin Monoclonal Antibody Binding to Thymic Sections. To assess the capacity of thymulin to inhibit the binding of antithymulin monoclonal antibodies to thymic sections, the monoclonal antibodies were preincubated with thymulin at 37°C for 30 min before being used in the IF assay. Synthetic thymulin was used at different concentrations (2×10^{-2} to 2×10^{-3} ng/μl). In some experiments, the antibody was preincubated with Chelex 100-treated thymulin or with Chelex 100-treated thymulin with $ZnCl_2$ (1 μM). Natural thymulin was used in the form of normal mouse or human serum (diluted 1:1 to 1:8) untreated or treated with Chelex 100. In addition, we used serum from patients with Di George syndrome, serum from children and mice with zinc deficiency, and supernatants from thymic or control (thyroid) epithelial cultures.

RESULTS

Functional Studies (Inhibition of Thymulin Biological Activity). *Several antithymulin antibodies suppress thymulin biological activity.* Incubation of synthetic thymulin with the different antithymulin antibodies blocked its biological activity. Similarly, the thymulin activity present in normal mouse or human serum and in thymic epithelial supernatants was abrogated by antithymulin monoclonal antibodies produced against the synthetic or natural hormone (2, 9, 10).

Table 2. Inhibitory effect of various antithymulin antibodies on thymulin biological activity (tested before and after zinc addition)

Treatment	Thymulin *in vitro* activity: Before zinc addition	Thymulin *in vitro* activity: After zinc addition
Synthetic thymulin		
None	$1–2 \times 10^{-6}$	$1–2 \times 10^{-6}$
Control antiserum	$1–2 \times 10^{-6}$	$1–2 \times 10^{-6}$
Antithymulin polyclonal antiserum	1–5	1–5*
Monoclonal antibody		
MA-FTS	1–5	1–5*
AS_1	1–5	$1–2 \times 10^{-5}$†
AE_1	1–5	$1–2 \times 10^{-5}$†
Control	$1–2 \times 10^{-6}$	$1–2 \times 10^{-6}$†
Natural thymulin‡		
None	1:128	1:128
Control antiserum	1:128	1:128
Antithymulin polyclonal antiserum	<1:4	<1:4*
Monoclonal antibody		
MA-FTS	<1:4	<1:4*
AS_1	<1:4	1:64†
AE_1	<1:4	1:64†
Control	1:128	1:128†

Thymulin samples were incubated with the antibody, the mixtures were filtered through an Amicon membrane, and the filtrate was tested in the rosette assay. Synthetic thymulin was used at a concentration of 1 μM. Results are expressed as the minimal active concentrations (ng/ml) or dilutions in the rosette assay. All experiments were performed in triplicate.
*Zinc-free and zinc-coupled thymulin remained unfiltered.
†Zinc-coupled thymulin was retained but zinc-free thymulin passed into the filtrate and could be reactivated after zinc addition.
‡Detected in normal mouse serum.

AS_1 and AE_1 antithymulin monoclonal antibodies specifically recognize zinc-containing thymulin. As described above, the monoclonal MA-FTS and the polyclonal antithymulin antibodies inhibited thymulin biological activity. However, when the peptide–antibody complexes (probably present in the synthetic preparation as a mixture of zinc-free and zinc-coupled molecule) were retained on an Amicon membrane, no hormonal activity was found in the filtrate even after $ZnCl_2$ addition, suggesting that all of the thymulin molecules had bound the antibody and, consequently, that the epitope(s) recognized by these antibodies is zinc independent. In contrast, if AS_1 and AE_1 monoclonal antibodies also inhibited thymulin biological activity, filtration of peptide–antibody complex did not prevent the passage of zinc-free peptide into the filtrate, as indicated by the hor-

Table 3. Specific capacity of AS_1 and AE_1 to recognize zinc-coupled thymulin, as measured by biological activity and IF assays

Incubation*	Inhibition of biological activity	Inhibition of fluorescence†
Chelex 100-treated thymulin	–	–
+ $ZnCl_2$	+	+
+ $Al_2Si_2O_7$	–	–
+ $CuSO_4$	–	–
+ $FeSO_4$	–	–

*Monoclonal antibodies were incubated with Chelex 100-treated thymulin and metal salts as indicated.
†Experiments performed in triplicate.

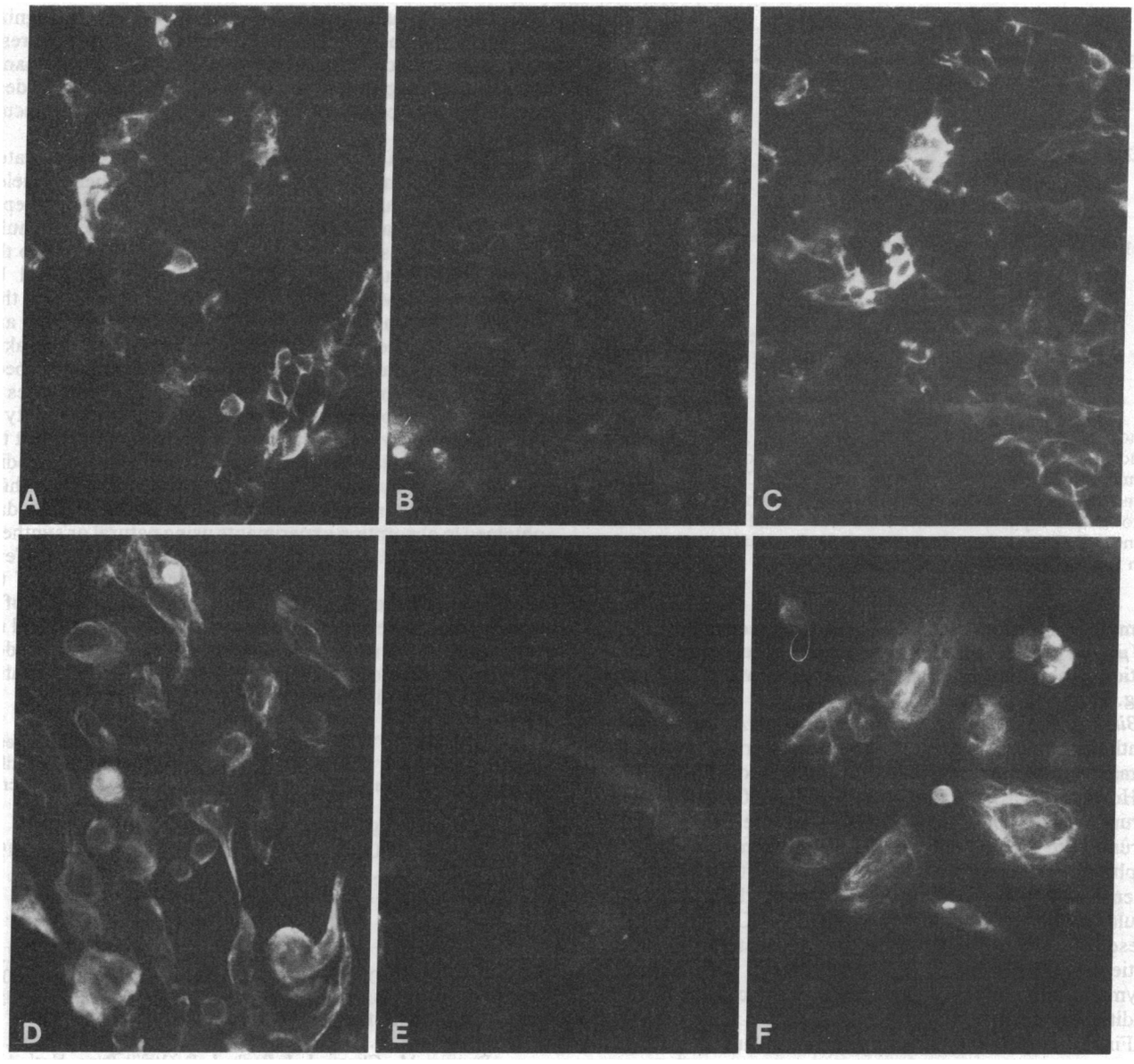

FIG. 1. Frozen sections of a normal human thymus (*A–C*) and cultured human TEC (*D–F*) immunolabeled with the AS_1 antithymulin monoclonal antibody, revealed by GAM/IgG1/FITC. In *A* and *D*, AS_1 was not pretreated and epithelial cells are clearly labeled. In *B* and *E*, AS_1 was preabsorbed with synthetic zinc-containing thymulin, which resulted in a complete fluorescence inhibition. In *C* and *F*, AS_1 was pretreated with Chelex 100-treated (zinc-free) thymulin, which did not inhibit the fluorescent labeling. (×450.) Similar results were obtained with the AE_1 antithymulin monoclonal antibody.

monal activity measured in the rosette inhibition assay after adding $ZnCl_2$. Nevertheless, this activity (10^{-5} ng/ml) was slightly lower than the initial activity (10^{-6} ng/ml). Finally, repeated absorptions of the peptide with the AS_1 and AE_1 antibodies followed by zinc addition resulted in the progressively decreased activity of the filtrate, which did not contain any significant activity after four absorptions. Similar results were obtained when using normal human or mouse serum, serum from zinc-deficient mice or humans (Table 2), and supernatants from human TEC cultures, indicating that these biological fluids also contain a mixture of zinc-free and zinc-coupled hormone (data not shown).

Similar absorption experiments were performed by using the peptide coupled to metals other than Zn^{2+}—namely, Fe^{2+}, Al^{3+}, and Cu^{2+}. These metals were selected because of their known capacities to provide some reactivation of the Chelex 100-treated molecule. As shown on Table 3, when Zn^{2+} was replaced by Fe^{2+}, Al^{3+}, or Cu^{2+} the biological activity of the hormone was no longer inhibited by these monoclonal antibodies, confirming the exclusive zinc dependency of their antibody activity. Conversely, the putative zinc-independent antithymulin polyclonal antiserum and the MA-FTS monoclonal antibody inhibited the biological activity of zinc-coupled as well as iron-, copper-, and aluminium-treated thymulin.

IF Studies. *Binding inhibition by synthetic thymulin.* As reported previously (9, 10), both AS_1 and AE_1 antithymulin monoclonal antibodies (but not MA-FTS) bound to murine and human TEC. The fluorescence could be inhibited completely by preincubation of the monoclonal antibodies with synthetic thymulin but not with Chelex 100-treated thymulin (Fig. 1). However, the addition of $ZnCl_2$ to Chelex 100-treated thymulin restored completely its ability to inhibit the fluorescence. Incubation of the monoclonal antibodies with $ZnCl_2$ alone had no effect (Table 3). The induced fluorescence inhibition was dependent on the peptide concentration.

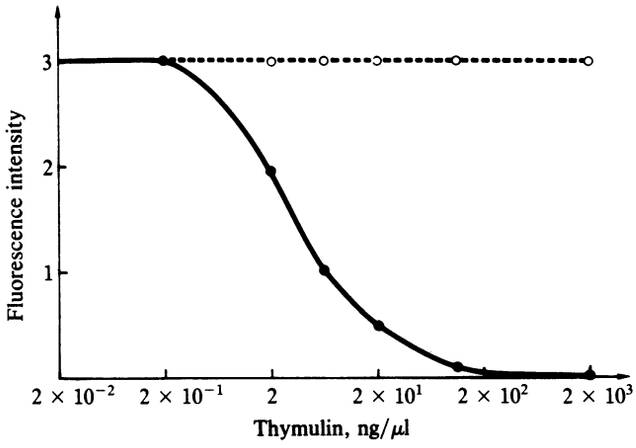

FIG. 2. Inhibition of fluorescent labeling of AE_1 antithymulin monoclonal antibody on human thymic sections by synthetic thymulin uncoupled or coupled to zinc. o--o, Chelex 100-treated thymulin; ●—●, zinc-containing thymulin. Each experiment was performed in triplicate. Studies were read "blind" and fluorescent intensity is expressed in arbitrary units. Similar results were obtained with the AS_1 antithymulin monoclonal antibody.

Complete inhibition was observed at concentrations $>10^2$ ng/μl and partial inhibition was observed at lower concentrations. Inhibition was no longer observed at 2×10^{-1} ng/μl (Fig. 2).

Binding inhibition by natural thymulin. As reported for synthetic thymulin, Chelex 100-treated normal mouse and human sera caused no fluorescence inhibition.

However, $ZnCl_2$ addition to the Chelex 100-treated normal serum restored their full inhibitory capacities. Interestingly, serum from zinc-deficient mice or children suffering from nephrotic syndrome with zinc deficiency left the fluorescent intensity virtually unchanged. However, the fluorescence could be almost completely abolished if $ZnCl_2$ was added to these sera. Conversely, serum from thymectomized mice or patients with Di George syndrome (known to be devoid of thymulin) did not cause any inhibitory effect even after the addition of $ZnCl_2$.

Finally, supernatants from cultures of human TEC also inhibited the fluorescence labeling, provided $ZnCl_2$ was added prior to the preincubation with the antithymulin antibodies. Thyroid culture supernatants (with or without $ZnCl_2$) tested as controls did not alter the intensity of the fluorescent labeling.

Absence of fluorescence inhibition by thymulin coupled to other metals. When synthetic and natural thymulin were chelated and then coupled to metals other than zinc (i.e., Fe^{2+}, Al^{3+}, or Cu^{2+}), they did not recover their capacities to inhibit the fluorescence obtained by the antithymulin monoclonal antibodies (Table 3).

DISCUSSION

The results described in this paper strongly suggest that at least one antigenic epitope in the thymulin molecule depends upon the presence of zinc. This epitope is selectively recognized by the antithymulin monoclonal antibodies AS_1 and AE_1. Other antibodies, such as the MA-FTS monoclonal antibody or some of the antibodies contained in the rabbit polyclonal antithymulin antiserum, produced against synthetic thymulin and screened by ELISA or RIA, recognize the peptide independently of the presence of zinc. Interestingly, the existence of zinc-dependent antigenic determinants has also been suggested for insulin (16). A metal-dependent epitope has also been reported for the prothrombin molecule (17).

The coupling of zinc to thymulin was directly demonstrated by studies showing that previously mixed ^3H-labeled Chelex 100-treated thymulin and radiolabeled zinc coelute in Sephadex G-25 chromatography (6) and the optimal zinc/thymulin molar ratio is close to 1 (8). The contribution of Zn^{2+} to the active conformation of thymulin is further indicated by spectroscopic NMR studies. These experiments showed that the conformations observed with the zinc-complexed and Chelex 100-treated peptide are clearly different.‡ Taken together, these data lead to the hypothesis that the epitope(s) recognized by the antithymulin monoclonal antibodies is directly related to the expression of the biological activity of thymulin. This hypothesis is in keeping with the fact that the two zinc-dependent antithymulin monoclonal antibodies used in this study were screened for their abilities to inhibit the zinc-dependent biological activity of thymulin. The data obtained in absorption experiments using natural or synthetic thymulin coupled to metals other than Zn^{2+}, such as Fe^{2+}, Al^{3+}, and Cu^{2+}, provide further evidence in favor of the recognition by the AS_1 and AE_1 monoclonal antibodies of an epitope strictly dependent upon the presence of zinc (and not other metals). Further experimentation is necessary to identify which amino acid(s) is involved in the association between zinc and the peptide.

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