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# Peroxidation potential of rat thymus during development and involution

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**The thymus of rats of ages between 1 and 7 months was homogenised and subjected to oxidative stress induced by iron salts. Lipid peroxidation, protein thiols and glutathione status were evaluated. The thymus of rats of 1 month of age exhibited lower susceptibility to the radical attack with respect to the thymus of rats between 3 and 7 months of age. This susceptibility was correlated with the content of polyunsaturated fatty acids and of lipophylic chain-breaking antioxidants.**

**Key words:** Thymus; Iron salts; Lipid peroxidation; Protein thiols; Glutathione; Antioxidants; Polyunsaturated fatty acids.

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## Introduction

The thymus is a complex organ deputed to proliferation and differentiation of mature T-lymphocytes with effector (cytotoxic) and regulatory (helper/suppressor) activities. The cellular components of the thymus are diversified according to their functions. In addition to the T cells at different maturation steps, epithelial cells do exist in the thymus capable of synthesizing thymic peptides acting on maturation and/or differentiation of stem cells (Goldstein, 1984). The thymus is the first organ to age and it is well known that, with advancing age, the gland undergoes a progressive involution both in size and in its physiological function. Thymus tissue is, as any other, subject to variation in oxygen tension, and to the attack of different toxic compounds; these situations may promote the generation of activated oxygen species of free radical character. It is conceivable that, during gland involution, the antioxidant defences may decrease or be sufficient only to protect against normal rates of radical generation and may not cope with increased rates. As a consequence, oxidative injury may occur, involving the direct oxidation of macromolecules essential for cell survival. In fact, lipid peroxidation, oxidative damage to proteins and DNA strand-breaks are well established features of oxidative stress found in many experimental systems (Slater, 1984; Halliwell and Gutteridge, 1989). As far as we know, we have little information regarding the susceptibility of rat thymus to undergo lipid peroxidation during gland development and involution. For this reason, it seemed worthwhile to investigate, in rats belonging to four different age groups, lipid peroxidizability, glutathione status and protein thiol oxidation of the whole tissue and to correlate the oxidative damage with the fatty acid composition and the content of chain breaking antioxidants.

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## Materials and Methods

All chemicals used were of analytical grade and were purchased from Merck (Darmstad, RFG). Solvents with HPLC or spectral grade (Merck).

Male Wistar rats between 1 and 7 months of age, which were allowed food and water *ad libitum*, were used. The animals were killed by decapitation and thymus glands, rapidly removed, were carefully homogenised with 9 vols

of 0.14 M NaCl/10 mM Mops, pH 6.5, in a Potter Elvehjem homogeniser at 0–4°C. The homogenate was then passed through two layers of cheese cloth to remove clumps.

In homogenates, lipid peroxidation was stimulated by iron salts. The reaction mixture (total vol: 1 ml) contained the same buffer used for homogenisation, protein (1 mg) $FeCl_2$ at different concentrations as specified in the figures. After 1 hr of incubation at 37°C, lipid peroxidation was measured by the formation of thiobarbituric acid reactive material (TBARS) according to a slightly modified procedure earlier described (Beuge and Aust, 1978). The incubated samples received, prior to TBA reagent, 10 $\mu$l of 2% (w/v) butylated hydroxytoluene (BHT) ethanolic solution and 200 $\mu$l of 8% (w/v) SDS. Then, 1.5 ml of a 20% (v/v) acetic acid solution, pH 3.5, and 1.5 ml of a 0.8% (w/v) TBA solution were added. The mixture was heated at 100°C for 15 min, cooled on ice and extracted with butanol. The organic phase was collected and read at 532 nm. Tetramethoxypropane solution yielding the TBA-adduct was used for calibration of absorbance data.

Total lipids were extracted according to Bligh and Dyer (1959). The fatty acid composition was determined by gas chromatography after transesterification of the lipid extracts (Christopherson and Glass, 1969) using a Carlo Erba HRGC 4160 model (Milano, Italy) equipped with a flame ionization detector and a fused silica capillary column (0.25 mm × 25 m; film of cyanopropylsilicone). The oven temperature was programmed from 140°C to 250°C at 5°C/min and the detector temperature was maintained at 280°C. Peaks were identified using standard fatty acid methylesters from Supelco (Bellafonte, PA, U.S.A.).

Lipophilic antioxidants such as $\alpha$-tocopherol ($\alpha$-T), coenzyme $Q_9$ ($Q_9$) and coenzyme $Q_{10}$ ($Q_{10}$) were separated by HPLC utilizing an Altex ODS 5 $\mu$m column (15 cm × 0.47 cm). The elution was isocratic at a flow rate of 1.5 ml/min with methanol/water (95:5, v/v) for determination of $\alpha$-T and methanol/isopropanol (85:15, v/v) for $Q_9$ and $Q_{10}$. Detection was at 291 nm and 275 nm for $\alpha$-T and coenzymes Q, respectively.

Protein was estimated after Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Protein sulphydryl groups were measured according to Di Monte *et al.* (1984). The reaction mixture (1 ml) treated with $FeCl_2$ was precipitated and washed twice with 6.5% (w/v) trichloracetic acid. The precipitate was solubilized in 300 $\mu$l 8% (w/v) SDS for 1–2 hr and finally suspended in 2 ml of 0.5 M Tris–Cl 5/mM EDTA, pH 7.6. The reaction was started by adding 200 $\mu$l of 2 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) and the increase of absorbance was followed at 412 nm until a constant value was obtained (about 5 min). The actual concentration of sulphydryl groups was calculated by using an $\epsilon_M = 13.600$. GSH levels were measured as acid-soluble thiols using the colorimetric assay of Akerboom and Sies (1981).

## Results and Discussion

Age-related changes of wet weight and cell number of the thymus of rats between 1 and 7 months of age, divided in four groups, are shown in Table 1. It is apparent that this lymphoid tissue reaches its maximum size at about 2–3 months of age, then it becomes much smaller during rat growth. The decrease in weight is due essentially to depletion of lymphocytes as confirmed by the data of Table 1. The involution of the thymus, in fact, is described as an event occurring after sexual maturation, the preceding period being characterized by a steady-state if not by a developmental growth stage. On the other hand, the architecture of the gland in old age resembles that of the young thymus, despite the fact that the older tissues are much smaller and embedded in fat tissue (Moccheggiani and Fabris, 1992; Steinman, 1986). Nevertheless, Table 1 shows that the lipid content related to thymus wet weight does not exhibit significant variations in the four groups of animals considered.

To investigate whether the susceptibility of this lymphoid tissue to undergo lipid peroxidation may vary with the age changes, thymus

Table 1. Influence of age on wet weight, cell number and lipid content of rat thymus

| Age (months) | Thymus wet weight* (g) | Cell number* ($\times 10^6$/thymus) | Lipids† (mg/g wet weight) |
|---|---|---|---|
| 1 | 0.37 ± 0.10 | 432 ± 51 | 13.90 ± 0.85 |
| 2–3 | 0.61 ± 0.03 | 816 ± 65 | 11.80 ± 0.20 |
| 4–5 | 0.20 ± 0.05 | 258 ± 33 | 11.95 ± 0.51 |
| 7 | 0.21 ± 0.09 | 56 ± 15 | 13.03 ± 0.55 |

*Data represent the mean ± SD of six thymus glands.
†Data represent the mean ± SD of three extracts of four thymus glands.

homogenates were subjected to oxidative stress. To this purpose, we used an experimental system which was previously set up to evaluate liposome peroxidation and to study selectively the propagation and termination phases of lipoperoxidation in particular (Tadolini *et al.*, 1989). The system has been applied also to induce peroxidation in microsomes (Tadolini *et al.*, 1992) and T-cells (Cabrini *et al.*, 1993). It involves the $Fe^{2+}$-promoted decomposition of lipid hydroperoxides (LOOH) in buffering conditions, where $Fe^{2+}$ autoxidation and oxygen active species generation do not occur. In these conditions, the rate and the extent of propagation chain reaction are dependent on the concentration of lipid peroxides.

As known, lipid peroxides are products of metabolic pathways *in vivo* and can be detected in body fluids and tissues (Yagi, 1982). In the absence of transition metals, specific enzymes, extreme heat, or irradiation, lipid peroxides remain intact. In animal systems, the transition metal complexes are the most likely threat to their stability. In the presence of iron and copper, in fact, they decompose to form peroxyl ($LOO^{\cdot}$) and alkoxyl ($LO^{\cdot}$) radicals, which can initiate lipid peroxidation by abstracting hydrogen atoms from unsaturated fatty acids (LH) according to the following equations:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-} \quad (1)$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\cdot} + H^{+} \quad (2)$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \quad (3)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (4)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}. \quad (5)$$

Therefore, the addition of $FeCl_2$ to peroxidizable substrates stimulated peroxidation only by a peroxide-decomposition reaction, provided that the buffer does not chelate iron ions and the pH is acidic (Tadolini *et al.*, 1989). In these experimental conditions, an estimate of the peroxidation potential of the biological system under investigation might be obtained.

When aliquots of thymus homogenates of rats of the four different ages reported in Table 1 were incubated at 37°C for 1 hr with increasing concentrations of $FeCl_2$, a biphasic pattern of TBARS formation was observed. As shown in Fig. 1, by at first increasing $FeCl_2$ concentration, TBARS formation increases progressively, reaches a plateau and then decreases. The homogenates between 3 and 7 months of age behave similarly, while the pattern of TBARS generation is significantly different at 1 month. The $FeCl_2$ concentration at which the production of lipid peroxidation end products is maximal is about 5 $\mu$M for the four preparations and it is dependent on homogenate

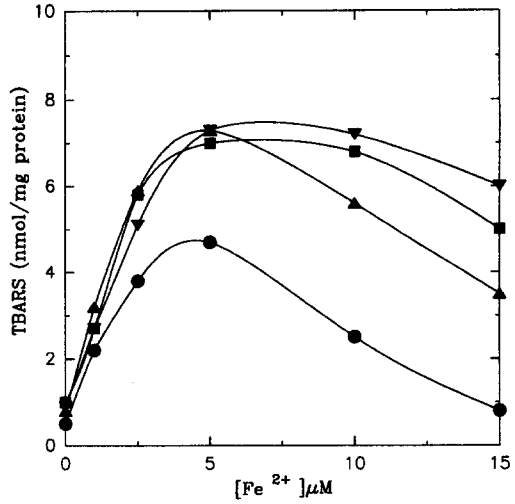


Fig. 1. TBARS generation in thymus homogenates of rats of different ages as a function of $FeCl_2$ concentration. Homogenates of 1 (●), 2–3 (▼), 4–5 (■) and 7 months (▲) of age. The experimental conditions were described in the Materials and Methods section. The values are means of three determinations.

amounts utilized (not shown). Furthermore, the results reported in Fig. 1 show that the maximum value of TBARS generation is considerably lower in the thymus of 1 month of age (4.8 nmol/mg protein) with respect to the other groups, in which about the same amount of TBARS is formed (7.5 nmol/mg protein).

As previously mentioned, the experimental protocol used allows one to point out the propagation step of lipoperoxidation. In fact, the addition of a chain-breaking antioxidant such as BHT exerted a strong inhibition on TBARS generation, while $OH^{\cdot}$ scavengers, such as mannitol and benzoate, did not affect lipid peroxidation (not shown).

It is well known that lipid peroxidation modifies also protein structure and function. Several membrane proteins derive some of their structure from closely associated lipids. During lipid peroxidation, two adjacent fatty acids can be joined by abnormal bonds, so that the structure may be altered and the activity affected. In addition, lipid peroxyl radicals can abstract hydrogen atoms from neighbouring proteins, resulting in lipid–protein and protein–protein cross-linking. Sulphydryl groups are, in general, highly reactive, and cellular protein thiols may represent critical targets in oxidative stress. Glutathione can also easily undergo oxidation of its SH group. GSH, as is well known, plays an important role among hydrophilic antioxidant systems. It functions both as a reductant in the metabolism of hydrogen peroxide and organic hydroperoxide and as a nucleophile which can

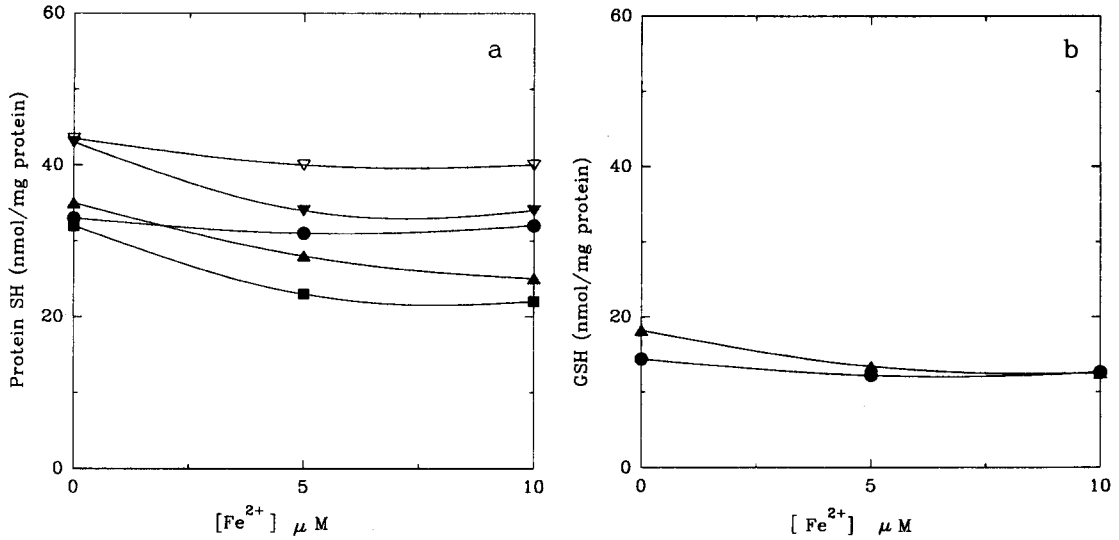


Fig. 2. Decrease of protein thiols and GSH in thymus homogenates of rats of different ages in the presence of iron ions. (a) Homogenates of 1 (●), 2–3 (▼), 4–5 (■) and 7 months (▲). Thymus homogenates of 3 months of age +0.04% (w/v) BHT (▽). (b) Thymus homogenates of 1 (●) and 7 months (▲) of age. The experimental conditions were described in the Materials and methods section. The values are means of three determinations.

conjugate electrophilic molecules (Cotgreave *et al.*, 1988; Meister, 1991).

We then investigated whether the decomposition of lipid peroxides, accelerated by iron ions, could affect protein sulphydryl groups and glutathione status of thymus homogenates. As shown in Fig. 2a, the decrease in protein thiols between 3 and 7 months of age was about 28% after 1 hr of incubation in the presence of both 5 and 10 $\mu$M $FeCl_2$, which are the iron ions concentrations able to generate the maximum value of TBARS. On the other hand, sulphydryl groups decreased by only 3% at 1 month of age in the same experimental conditions, and a classical chain-breaking antioxidant such as BHT was able to inhibit the disappearance of protein thiols (Fig. 2a), suggesting that protein-SH oxidation is consequent to lipid peroxidation.

Lipid peroxidation determined by the decomposition of lipid hydroperoxides resulted also in glutathione decrease, which was only 12% in the thymus of 1 month of age and 33% at 7 months (Fig. 2b).

In our experimental conditions, the susceptibility of the whole thymus to the oxidative stress can be dependent on the degree of lipid unsaturation, which can lead to the formation of small amounts of lipid peroxides, the substrate of decomposition catalyzed by iron ions. Thus, the fatty acid composition of the lipid extract of the four thymus homogenates was determined. From data reported in Table 2, it is apparent that the thymus of 1 month of age exhibits a lower ratio of polyenoic to saturated plus monoenoic fatty acids with respect to the three other groups. This fact can partially explain the lower value of TBARS formation and consequently the lower decrease of sulphydryl groups both in proteins and GSH at this age. On the other hand, the relative susceptibility of rat thymus of different ages to lipid peroxidation can also be correlated to the content of lipophilic antioxidants such as $\alpha$-tocopherol and coenzyme Q, which, being hydrophobic, will tend to concentrate in the interior of membranes. Chain-breaking antioxidants, scavenging intermediate radicals such as peroxyl and alkoxyl radicals, can prevent continued

Table 2. Effects of age on fatty acid composition of rat thymus total lipids

| | Age (months) | | | |
|---|---|---|---|---|
| Fatty acids | 1 | 2–3 | 4–5 | 7 |
| 14:0 | 1.5 | 2.0 | 1.9 | 1.2 |
| 16:0 | 29.0 | 28.0 | 25.4 | 26.0 |
| 16:1 | 5.7 | 4.4 | 4.7 | 2.3 |
| 17:0 | 1.0 | 1.5 | 1.3 | 0.6 |
| 18:0 | 8.7 | 9.3 | 7.6 | 10.6 |
| 18:1 | 24.5 | 17.6 | 22.9 | 17.8 |
| 18:2 | 20.0 | 23.7 | 25.8 | 26.5 |
| 20:4 | 8.7 | 11.6 | 8.9 | 12.2 |
| 22:4 | 0.9 | 1.2 | 1.1 | 1.1 |
| 22:6 | 0.5 | 0.3 | 0.3 | 0.5 |
| P/S | 0.43 | 0.58 | 0.56 | 0.66 |

Data represent the mean of three gas chromatographic analysis.
The fatty acid composition is expressed as a percentage of the total identified fatty acids.
P/S = polyunsaturated to saturated + monounsaturated fatty acids ratio.

Table 3. Influence of age on the content of lipophilic antioxidants of rat thymus

| Age (months) | α-Tocopherol (nmol/g wet weight) | Coenzyme $Q_9$ (nmol/g wet weight) | Coenzyme $Q_{10}$ (nmol/g wet weight) |
|---|---|---|---|
| 1 | 6.95 ± 0.15* | 10.29 ± 0.46† | 4.45 ± 0.37† |
| 2–3 | 5.66 ± 0.20 | 10.97 ± 0.20 | 3.54 ± 0.40 |
| 4–5 | 3.38 ± 0.10 | 9.12 ± 0.40 | 3.05 ± 0.38 |
| 7 | 3.64 ± 0.12 | 8.86 ± 0.45 | 2.74 ± 0.45 |

Data represent the mean ± SD of three extracts of four thymus glands.
*$P < 0.001$ vs 4–5 and 7 months.
†$P < 0.05$ vs 4–5 and 7 months.

hydrogen abstraction by donating labile hydrogen to them and so terminating the chain reaction of peroxidation. The activity of these antioxidants has to be particularly considered in the peroxidation system used here, which is capable of investigating the propagation step of lipoperoxidation, as previously pointed out.

Vitamin E is the major lipophilic antioxidant. Coenzyme Q, in addition to its well known role as redox component of the mitochondrial electron transport system involved in energy conservation, functions, in its reduced form, as an antioxidant *in vitro* (Mellors and Tappel, 1966; Frei *et al.*, 1990; Landi *et al.*, 1992) and *in vivo* (Sugino *et al.*, 1989; Ernster and Beyer, 1991).

This evidence might explain its broad distribution in subcellular membranes (Appelkvist *et al.*, 1991). In mammals, the most common forms of Q contain 9 ($Q_9$) and 10 ($Q_{10}$) isoprene units (Crane, 1965), which coexist in different amounts in different mammalian species (Takada *et al.*, 1984).

HPLC determination of the three lipophilic compounds reported in Table 3 clearly shows that: (i) the content of coenzyme Q is always higher than that of α-T; and (ii) the thymus of 1 month of age exhibits, in addition to the lower ratio of polyunsaturated to saturated fatty acids, also a higher content of lipophilic chain-breaking antioxidants. In fact, the amounts of α-T and total Q are 6.95 and 17.74 nmol/wet weight, respectively, in the gland of 1 month of age. These contents are nearly equivalent at 2–3 months but significantly lower in the thymus of older rats. This can be due to the depletion of lymphocytes during thymus involution. Our recent results (Cabrini *et al.*, 1993) have shown, in fact, that the concentration of chain breaking antioxidants in thymocytes is higher than those found in the whole tissue.

In conclusion, the data here reported suggest that the susceptibility of rat thymus to oxidative stress is different depending on the age of the animal. In fact, both lipid peroxidation and the consequent oxidation of sulphydryl groups is very low in the thymus of rats of 1 month of age with respect to those of adult rats. These findings might be ascribed to a lower unsaturation index and to a higher content of lipophilic antioxidant of the young tissue. Nevertheless other defense mechanisms, not examined in this work, may also be involved in removing peroxides, e.g. glutathione peroxidase, or in binding metal ions in form that do not generate reactive species. At present, it is difficult to assign a physiological meaning to the different peroxidizability of the whole thymus during rat growth. A working hypothesis might be that the low susceptibility of the thymus to lipid peroxidation in early life could be related to its essential activities of controller and "educator" of the various subset of T cells.

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