

## EFFECTS OF ALPHA INTERFERON ON THE LYMPHOID TISSUE OF THE SPLEEN IN YOUNG ADULT AND SENILE MICE

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### INTRODUCTION

Interferons are natural cytokines with a variety of biological effects including antiviral, antiproliferative, antifibrogenic and antianigenic properties (Hiramatsu et al., 1995; Muriel 1996; Kim et al., 2000). Interferons inhibit transcription and translation of a number of RNA and DNA viruses as well as the growth and proliferation of a variety of cells (Theocharis et al., 1997). Furthermore, interferons could alleviate fibrosis and inflammation (Mazzoran et al., 1998). The two main members of interferon I family namely interferon alpha and beta have gained special value as they proved to be successful in treatment of chronic viral infections, myoproliferative disorders and autoimmune diseases. Interferon alpha is the only available effective treatment for chronic viral hepatitis C (Kaserer et al., 1998; Duchatelle et al., 1998). It is also used in chronic hepatitis B, chronic non A non B hepatitis and chronic delta hepatitis (Yoo et al., 1990; Kleiner et al., 1993; Manabe et al., 1993). Moreover, it is useful in reversing liver damage induced by biliary obstruction (Moreno & Muriel 1995). Through its immunomodulatory activity, interferons decrease the incidence of severity of precancerous lesions (Merle et al., 1997). It is also efficacious in treatment of pancreatic tumors, renal cell carcinoma, chronic myelogenous leukemia, hairy cell leukemia, cutaneous melanoma and polycythemia (Lamers 1990; Baer et al., 1992; Toliou et al., 1996; Bose et al., 1997; Elliot & Tefferi 1997; Otely & Zitelli 2000).

Despite of the variation in the etiology of the diseases treated by interferons and the different types of immune responses thought to be required to control them, interferon I is able to provide effective therapy. It is likely that interferon induced marked changes in the immune status which depend on its ability to influence the

differentiation of B and T lymphocytes and increase lymphocyte adhesion to endothelial cells by up-regulation of adhesion molecules (Steinger & Van Der Meide, 1993). Among its other immunological effects, interferons provoked activation and proliferation of monocytes, macrophages, cytotoxic T cells and natural killer cells eliciting long lasting tumor specific immunity (Eguchi et al., 2003). These changes were accompanied by augmented expression of surface activation markers on the effector cells (Suzuki et al., 2002).

Reviewing the literature demonstrated that alpha interferons inhibited the spontaneous apoptosis of primary B lymphocytes and promoted survival of peripheral B lymphocytes enhancing immune responses (Ruuth et al., 2001). Few data, however are available about the morphological changes induced by alpha interferon, the different phases of cell cycle and DNA content in lymphocyte populations of the different lymphoid organs.

On the other hand, several factors were identified to influence and limit the response to interferon therapy negatively. Among these factors are the long duration of the disease, the elevated histological indexes, the high levels of the virus, alcoholism, and the high age of the patients (Ono et al., 1996; Kaserer et al., 1998). Aging in particular is associated with dysfunction of immune system and deterioration of immunity. Dramatic decline of pre-B cells number and reduced proliferative capacity of both T and B lymphocytes as well as the germinal centers are common consecutive changes of aging (Johnson et al., 2002; Blasco, 2002). Moreover, the process of aging has been proposed to be associated with gradual accumulation of destructive products of endogenously generated free radicals. The consequences of endogenously generated radicals are usually estimated in terms of damage to macromolecules most notably lipids, proteins and DNA (El-Sokkary et al., 2003).

Hence, it becomes the aim of the present work to clarify the senile changes that are likely to occur in the spleen of mice. Furthermore, the present study aimed at investigating and comparing the morphological changes, the phases of the cell cycle and the DNA content in adult and senile spleen following interferon alpha administration. It is believed that the spleen is a major repository of immune cells in the body that can reflect any changes in the immune status. The evaluation of immunological status before initiation of treatment might be of great clinical importance as it has a major influence on the effects and outcome of interferon therapy.

## MATERIAL AND METHODS

### **Mice :**

Forty CBA male mice locally bred at the animal house of the Research Unit and Bilharzial Research Center of Faculty of Medicine, Ain Shamus University were used in the present study. Adult mice aged 3 - 4 months and weighed 25 - 30 grams. Senile mice aged 18 - 24 months and weighed 30 - 35 grams. Animals were housed in separate cages, fed on standard mice diet and allowed water ad-libitum.

### **Interferon :**

Alpha interferon was available as vials of "Interon A, injection" (Schering Phough USA). Each vial contained 3 million IU of interferon Alpha 2b. Vials were reconstituted with 1 ml of sterile water and stored at temperature 2 - 8c°, each mice received  $3.3 \times 10^4$  IU / kg B.W. Interferon was diluted to the appropriate concentration in sterile water of sufficient volume for a single dose for each group of mice. The dose and regimen given were equivalent and similar to human therapeutic dose (Theocharis et al., 1997). At the beginning of the experiment mice were divided into four groups as follows :

### **Group I :**

It included five adult mice and served as adult controls to study the histological structure of adult spleen.

### **Group II :**

It included five senile mice and served as senile controls to study the age related changes in the spleen.

### **Group III :**

It included 15 adult mice of which ten mice received 1000 IU of interferon alpha subcutaneously. The dose was given three times a week for three months. Five mice received equivalent sterile water, injected subcutaneously for the same duration and served as controls.

### **Group IV :**

It included 15 senile mice of which ten mice received 1200 IU of interferon alpha subcutaneously. The dose was given three times a week for three months. Five

mice received equivalent sterile water, injected subcutaneously for the same duration and served as controls.

At the end of the experiment all experimental animals were sacrificed by intraperitoneal injection of sodium thiopental (25 mg / kg). The abdomen was incised and the spleens were dissected and extracted. Specimens for light microscopic studies were fixed in 10% formol saline. After fixation, tissues were dehydrated, and embedded in paraffin blocks. Sections of 5  $\mu\text{m}$  in thickness were cut and stained with Haematoxylin and Eosin (Hx. & E.) and Feulgen blue stain specific for nuclear DNA. Specimens for electron microscopic studies were cut into small pieces 1  $\text{mm}^3$  in thickness, fixed in 2.5% glutaraldehyde in phosphate buffer for 3 hours. Fixed tissues were washed in 0.1 M-phosphate buffer and post fixed in 1% osmium tetra oxide in 0.1 M-phosphate buffer. Dehydration in ascending grades of alcohol was followed by embedding in epon. Semithin sections 1  $\mu\text{m}$  were cut using L.K.B. ultramicrotome and stained with toluidine blue. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, examined and photographed with Philips 400 T.E.M.

#### **Image analysis :**

The DNA was analyzed at the pathology department of the National Research Center using the Leica Qwin 500 Image Analyzer. DNA ploidy analysis was performed on the Feulgen blue stained sections using the DNA cytometry soft ware. Selection of nuclear boundaries was performed automatically by the image analysis system. Only intact nuclei were measured, overlapping nuclei and nuclear fragments were manually eliminated from measurements. The optical density of the selected nuclei was measured and automatically converted by the system into DNA content. About 100 - 150 nuclei were measured in serial sections on magnification (x 400). The results were displayed as a frequency histogram on the monitor by plotting DNA content versus the number of nuclei. Based on the peaks produced by the histograms, the percentage of cells at each phase of cell cycle and their DNA content were calculated automatically. The results were analyzed using student t-test to compare the mean values and Z test compare the percentage of the cells.

## **RESULTS**

#### **Group I & Controls of Group III :**

Light microscopic examination of the spleen of adult mice revealed that the spleen exhibited prominent discrete white pulps suspended throughout the red pulp.

Distinct demarcation was evident between the white and red pulps. The white pulp of the spleen existed in three discrete zones namely the periarteriolar lymphatic sheath (PALS), the lymphatic follicles and the marginal zone. The PALS consisted of closely packed small lymphocytes surrounding the arterioles in a rather regular pattern. The lymphatic follicles were rounded or oval aggregates of small lymphocytes. The lymphocytes manifested dense rounded nuclei and relatively small amount of cytoplasm. Both the (PALS) and the lymphatic follicles were surrounded by a marginal zone which appeared as a pale thin rim of less densely packed small lymphocytes with few interposed large lymphocytes (Fig. 1). The red pulp appeared as irregular cords of red blood cells mingled with few lymphocytes. The cords were separated by venous sinuses (Fig. 2).

Ultrastructural examination clarified that small lymphocytes were predominant in the PALS and the lymphatic follicles. Small lymphocytes were characterized by high nuclear cytoplasmic ratio. The nuclei were rounded or oval in shape with prominent nucleolus and moderately condensed chromatin which appeared as coarse clumps. The cytoplasm was sparse and contained very few mitochondria and comparatively large number of poly-ribosomes (Fig. 3). Large lymphocytes were noticed mainly in the marginal zone. They had indented nuclei with peripherally condensed chromatin and rounded nucleolus. The cytoplasm was relatively abundant and contained numerous rounded mitochondria, few cisterns of rough endoplasmic reticulum, Golgi complex and numerous polyribosomes distributed throughout the cytoplasm (Fig. 4).

#### **DNA Analysis of Group I & Controls of Group III : (Tables 1, 2)**

DNA analysis of Feulgen blue stained sections of the spleen of adult mice, revealed that the majority of lymphocytes (75.22%) were at G<sub>1</sub> phase (presynthesis phase) of the cell cycle. The mean value of DNA was  $1.94 \pm 0.26$ . The percentage of lymphocytes at S phase (synthesis phase) of cycle was 16.51% showing a medium proliferative index. Their mean DNA value was  $2.85 \pm 0.29$ . On the other hand few lymphocytes (6.42%) were at G<sub>0</sub> phase (arrested phase) with a mean value of DNA  $1.43 \pm 0.5$ . Furthermore very few lymphocytes (0.92%) were noticed at G<sub>2</sub> phase (post duplication phase) with a mean value of DNA  $3.63 \pm 0$ . Other lymphocytes (0.92%) were at the aneuploid phase with a mean value of DNA  $4.515 \pm 0$ .

### **Group II & Controls of Group IV :**

Light microscopic examination of the spleen of senile mice showed marked regression of the white pulps throughout the prominent red pulps. The loss of lymphoid cells was apparent in both white and red pulps. Moreover, the distinct demarcation between the zones of white pulp was ill defined (Figs. 5, 6 & 7). The PALS consisted of sparse lymphocytes that surrounded the arterioles in an irregular pattern. The arteriolar wall exhibited multiple vacuolation involving the entire circumference, irregular endothelial lining and marked thickening of the muscle layer (Fig. 6). The lymphatic follicles were either shrunken or irregular in shape and exhibited marked depletion of lymphocytes. Marginal zones no longer existed as a discrete compartment that could be distinguished from the red pulp and it appeared almost devoid of lymphocytes (Figs. 6, 7). The red pulp contained numerous extensive red blood cells, located within dilated sinuses. Meanwhile, the red pulp showed depletion of lymphocytic cells (Fig. 7).

Ultrastructural examination clarified that lymphocytes manifested various forms of degeneration. Small lymphocytes though exhibited rounded nuclei with peripherally condensed chromatin, yet their cytoplasm was degenerated and ill defined. The plasmalemma was disrupted and the lymphocytes lacked the distinct cellular boundaries (Fig. 8). On the other hand, large lymphocytes manifested marked degenerative changes mainly in the cytoplasm and to a lesser extent in the nuclei. In the majority of the lymphocytes the cytoplasm presented multiple vacuolar degeneration, irregular in shape and variable in size (Fig. 9). The cytoplasmic organelles were ill defined apart from few scattered lysosomes (Fig. 10). Other lymphocytes showed in addition to the cytoplasmic degeneration, osmiophilic, pyknotic nuclei, reduced in size and with totally condensed chromatin (Figs. 10, 11). Moreover, the degenerated lymphocytes showed large cytoplasmic blebs or vesicles protruding from the cell surface and enclosed by a plasmic membrane (Fig. 11).

### **DNA analysis of Group II & controls of Group IV : (Tables 1, 2)**

DNA analysis of the Feulgen blue stained sections of the senile mice revealed that the majority of lymphocytes (66.34%) were at G<sub>1</sub> phase showing non significant variation than adult mice ( $P > 0.05$ ). Similarly the mean value of DNA was  $1.97 \pm 0.280$  manifesting non significant variation than adult mice ( $P > 0.05$ ). The percentage of cells at S phase was 18.26% showing a medium proliferative index and non significant variation than adult mice ( $P > 0.05$ ). The mean value of DNA was  $2.807 \pm 0.230$  showing again non significant variation than adult mice ( $P > 0.05$ ).

On the other hand the percentage of cells at G<sub>0</sub> phase (15.38%) was significantly increased ( $P < 0.05$ ) and the mean DNA value ( $1.302 \pm 0.191$ ) was significantly reduced ( $P < 0.01$ ). No lymphocytes could be detected at G<sub>2</sub> phase nor at the aneuploid phase of cell cycle.

### **Group II :**

Light microscopic examination of the spleen of adult mice which received interferon revealed that the spleen exhibited impressive, prominent white pulps even encroaching on the regressed red pulp. The distinct zones of the white pulp were amalgamated together forming a confluent mass of lymphocytes (Fig. 12). The degree of condensation of lymphocytes was variable, some areas showed highly condensed and closely packed lymphocytes, while other areas manifested loosely packed lymphocytes (Fig. 13). Most of the lymphocytes had large dark basophilic nuclei surrounded by a narrow rim of cytoplasm (Fig. 12). The red pulp contained few scattered red blood cells and obliterated sinuses (Figs. 13, 14). The characteristic finding was the presence of clusters of lymphocytes with dark basophilic nuclei and narrow rim of cytoplasm, invading the red pulp (Fig. 13). Numerous megakaryocytes were noticed infiltrating the white pulp. The megakaryocytes were large giant cells which exhibited irregular multilobed nuclei and extensive cytoplasm (Figs. 13, 14).

Ultrastructural examination, clarified signs of activation, and proliferation in the majority of the lymphocytes. The nuclei exhibited either enlarged, dispersed and homogenous chromatin (Fig. 15) or manifested loss of nuclear membrane, nucleolus and in such cases the chromatin, appeared as multiple dense blocks that were arranged approximately on either sides of the cell (Fig. 16). The cytoplasm had striking extensive proliferation of the rough endoplasmic reticulum which occupied the entire of the cytoplasm. The rough endoplasmic reticulum was concentrically arranged and consisted of regular cisterns almost of uniform diameter apart from few slightly dilated cisterns (Fig. 17). Other lymphocytes manifested partially euchromatic nuclei their cytoplasm contained few dilated cisterns of rough endoplasmic reticulum, scattered mitochondria and numerous free ribosomes (Fig. 18). Occasionally, the present study revealed that the plasmic membranes of some lymphocytes were opposed and adherent at localized sites but without specific cytological specialization in the area of contact (Fig. 19).

### **DNA analysis of group III : (Tables 1, 2)**

DNA analysis of Feulgen blue stained sections of spleen of adult mice which received interferons revealed prominent alterations. The majority of lymphocytes (55.66%) were at S phase showing high proliferative index and high significant increase than adult control ( $P < 0.001$ ). Moreover, the mean DNA value was  $3.032 \pm 0.274$  manifesting also high significant increase than the adult control ( $P < 0.01$ ). The percentage of lymphocytes at  $G_2$  phase (23.59%) was significantly increased than adult control ( $P < 0.001$ ) and their mean DNA value was  $3.86 \pm 0.198$ . The lymphocytes at  $G_1$  phase were significantly reduced (16.04%  $P < 0.001$ ). However, the mean DNA value was  $2.152 \pm 0.22$  showing significant increase than adult control ( $P < 0.05$ ). Aneuploid phase cells were significantly increased to 4.72% ( $P < 0.01$ ), their mean DNA value  $5.408 \pm 0.682$ . On the other hand, lymphocytes at  $G_0$  could not be detected and showed high significant decrease ( $P < 0.01$ ).

### **Group IV :**

Light microscopic examination of the spleen of senile mice which received interferons clarified distorted splenic architecture. Multiple areas of depletion were encountered in the white pulp and numerous extensive vacuolation was noticed in the red pulp. The lymphocytes were diffusely scattered without discrimination into zones (Figs. 20, 21). In spite of the encountered depletion, the existing lymphocytes had large prominent basophilic nuclei surrounded by a rim of cytoplasm (Fig. 20). Megakaryocytes were infiltrating both white and red pulps (Figs. 20, 21). The red pulp consisted only of few red blood cells irregularly arranged between numerous irregular vacuoles (Fig. 21).

Ultrastructural studies revealed that the small lymphocytes had intact nuclei, and their cytoplasm showed numerous ribosomes. However, small lymphocytes exhibited slight widening of the perinuclear space (Fig. 22). Regarding large lymphocytes, the nuclei had peripherally condensed chromatin, the cytoplasm contained multiple, dilated cisterns of rough endoplasmic reticulum scattered within the cytoplasm. The dilated cisterns showed partial detachment of ribosomes (Fig. 22). Other cells exhibited slight proliferation of the rough endoplasmic reticulum (Fig. 23).

### **DNA analysis of group IV : (Tables 1, 2)**

DNA analysis of Feulgen blue stained sections of senile mice which received interferons clarified that the majority of lymphocytes (47.53%) were at S phase with a high proliferative index. The percentage of lymphocytes at S phase was



significantly higher than senile control ( $P < 0.001$ ). Moreover, the mean DNA value was  $2.998 \pm 0.273$  significantly increased than senile controls ( $P < 0.01$ ). The percentage of lymphocytes at  $G_2$  phase (21.78%) was significantly higher than senile controls ( $P < 0.001$ ) and the mean DNA was  $4.010 \pm 0.317$ . The lymphocytes at  $G_1$  phase showed highly significant decrease (20.79%  $P < 0.001$ ). However, their DNA mean value was  $2.165 \pm 0.217$  showing highly significant increase than senile controls ( $P < 0.01$ ). The aneuploid phase cells were about 9.90% showing significant increase ( $P < 0.001$ ) than senile controls. Their mean DNA value was  $5.681 \pm 1.707$ . The percentage of lymphocytes at  $G_0$  phase manifested highly significant decrease ( $P < 0.001$ ).

**Table 1 : Percentage of Lymphocytes in Different Phases of Cell Cycle :**

	Percentage of cells In different phases of cell cycle	Adult Spleen	Senile Spleen
Adult Spleen	G <sub>0</sub> : 6.42% G <sub>1</sub> : 75.22% S : 16.51% G <sub>2</sub> : 0.92% Aneuploid 0.92%		
Senile Spleen	G <sub>0</sub> : 15.38% G <sub>1</sub> : 66.34% S : 18.26% G <sub>2</sub> : Aneuploid :	Z: 2.06 - P<0.05 S Z: 1.43 - P>0.05 N.S Z: 0.33 - P>0.05 N.S Z: -0.96 - P>0.05 N.S Z: -0.96 - P>0.05 N.S	
Adult spleen following interferon	G <sub>0</sub> : G <sub>1</sub> : 16.04% S : 55.66% G <sub>2</sub> : 23.59% Aneuploid 4.72%	Z: 2.63 - P<0.01 HS Z: 8.57 - P<0.001 H.S Z: 5.88 - P<0.001 H.S Z: 4.99 - P<0.001 H.S Z: 1.66 - P<0.01 H.S	
Senile spleen following interferon	G <sub>0</sub> : G <sub>1</sub> : 20.79% S : 47.53% G <sub>2</sub> : 21.78% Aneuploid 9.90%		Z: 4.16 - P<0.001 H.S Z: 6.63 - P<0.001 H.S Z: -4.52 - P<0.001 H.S Z: -5.11 - P<0.001 H.S Z: -3.34 - P<0.001 H.S

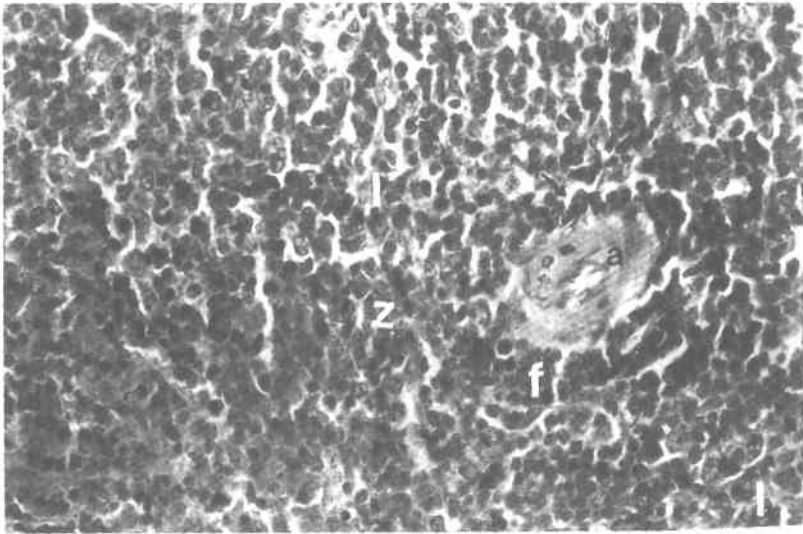
HS : Highly Significant.

S : Significant.

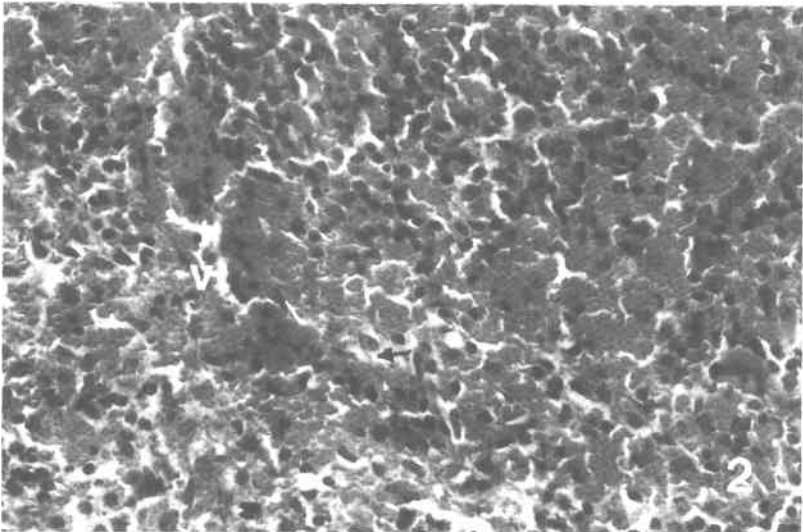
NS : Non-Significant.

**Table 2 : DNA Content of Lymphocytes in Different Phases of Cell Cycle :**

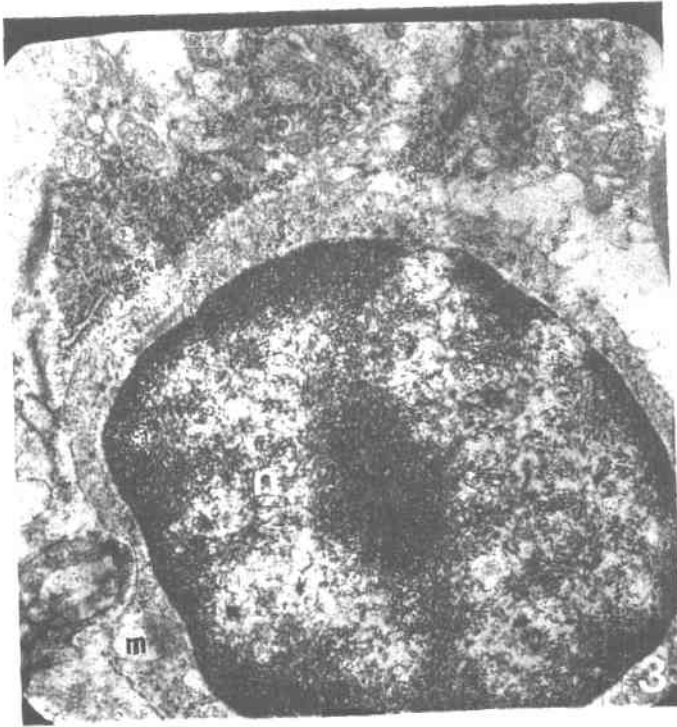
	DNA content in phases of cell cycle M ± SD	Adult Spleen	Senile Spleen
<b>Adult Spleen</b>	G <sub>0</sub> : 1.43 ± 0.5 G <sub>1</sub> : 1.94 ± 0.26 S : 2.85 ± 0.29 G <sub>2</sub> : 3.63 ± 0 Aneuploid 4.515 ± 0		
<b>Senile Spleen</b>	G <sub>0</sub> : 1.302 ± 0.191 G <sub>1</sub> : 1.97 ± 0.280 S : 2.807 ± 0.230 G <sub>2</sub> : Aneuploid :	t: -2.54 - P<0.01 HS t: 9 - P>0.05 N.S t: -46 - P>0.05 N.S	
<b>Adult spleen following interferon</b>	G <sub>0</sub> : G <sub>1</sub> : 2.152 ± 0.22 S : 3.032 ± 0.274 G <sub>2</sub> : 3.86 ± 0.198 Aneuploid 5.408 ± 0.682	t: 3.104 - P<0.05 S t: 2.434 - P<0.01 H.S	
<b>Senile spleen following interferon</b>	G <sub>0</sub> : G <sub>1</sub> : 2.165 ± 0.217 S : 2.988 ± 0.273 G <sub>2</sub> : 4.010 ± 0.317 Aneuploid 5.681 ± 1.707		t: -3.24 - P<0.01 H.S t: -2.90 - P<0.01 H.S



**Fig. (1) :** A photomicrograph of a section of the spleen of an adult mouse showing the arteriole (a) surrounded by (PAI.S) (↑) lymphatic follicle (f) and marginal zone (z). Notice the large lymphocytes (l).  
(Hx. & E.; x400)



**Fig. (2) :** A photomicrograph of a section of the spleen of an adult mouse showing the cords of red blood cells in the red pulp (r). Notice the venous sinuses (v).  
(Hx. & E.; x400)



**Fig. (3) :** An electron micrograph of the spleen of an adult mouse illustrating a small lymphocyte with rounded nucleus (n), prominent nucleolus and narrow rim of cytoplasm. Notice the mitochondria (m).  
(Uryl acetate & lead citrate; x 10,000)

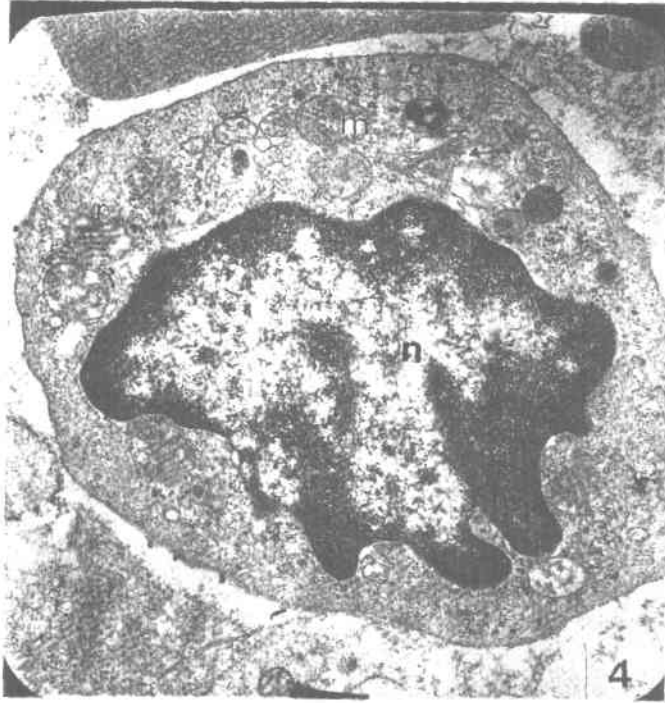


Fig. (4) : An electron micrograph of the spleen of an adult mouse showing a large lymphocyte with indented nucleus (n). The cytoplasm has mitochondria (m), cisterns of rough endoplasmic reticulum (r), Golgi complex (G) and numerous ribosomes.  
(Uranyl acetate & lead citrate; x 10.000)

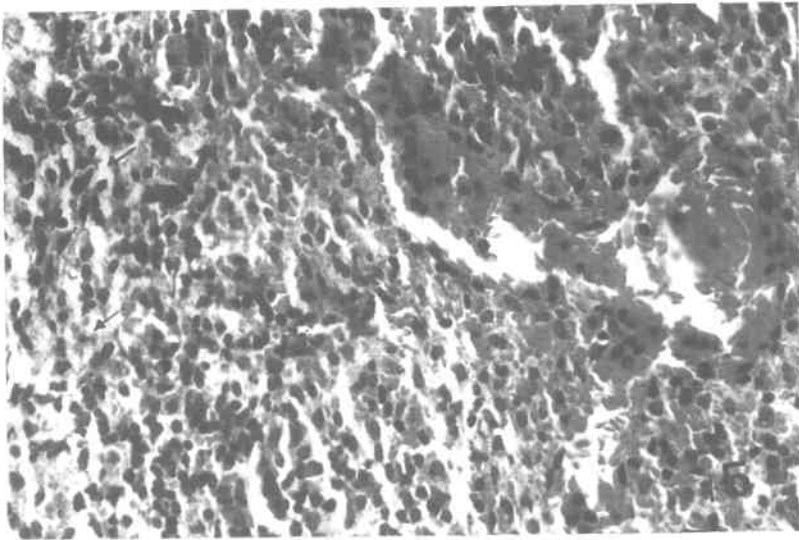


Fig. (5) : A photomicrograph of a section of the spleen of a senile mouse showing loss of lymphoid cells (↑) and loss of demarcation between red and white pulps.  
(Hx. & E.; x400)

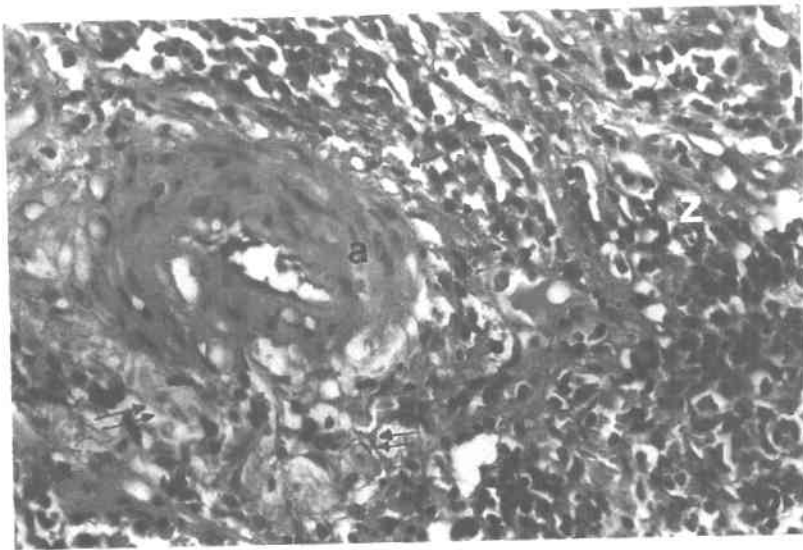
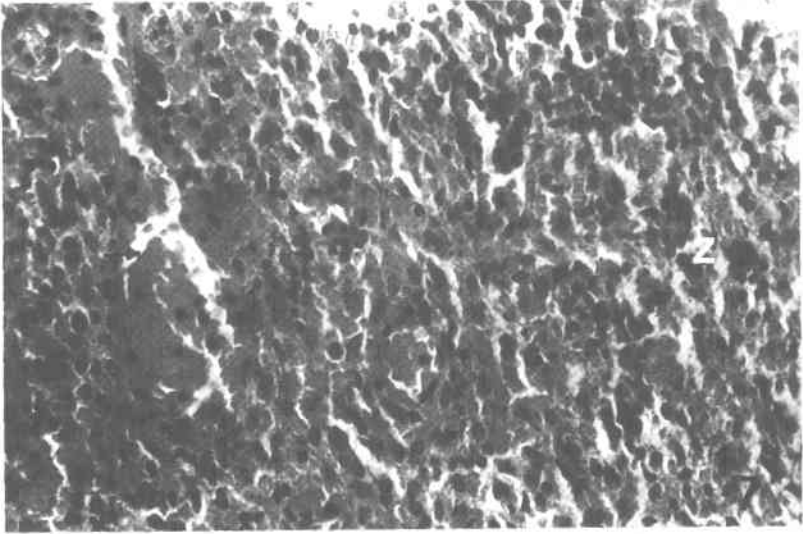


Fig. (6) : A photomicrograph of a section of the spleen of a senile mouse demonstrating the arteriole (a) with multiple vacuoles and thickened muscle layer. Notice the depletion of lymphocytes in (PALS) (↑), lymphatic follicles (↑↑) and the marginal zone (z).  
(Hx. & E.; x400)



**Fig. (7)** : A photomicrograph of a section of the spleen of a senile mouse revealing extensive red blood cells (↑). Notice the ill-defined marginal zone (z).

(Hx. & E.; x400)



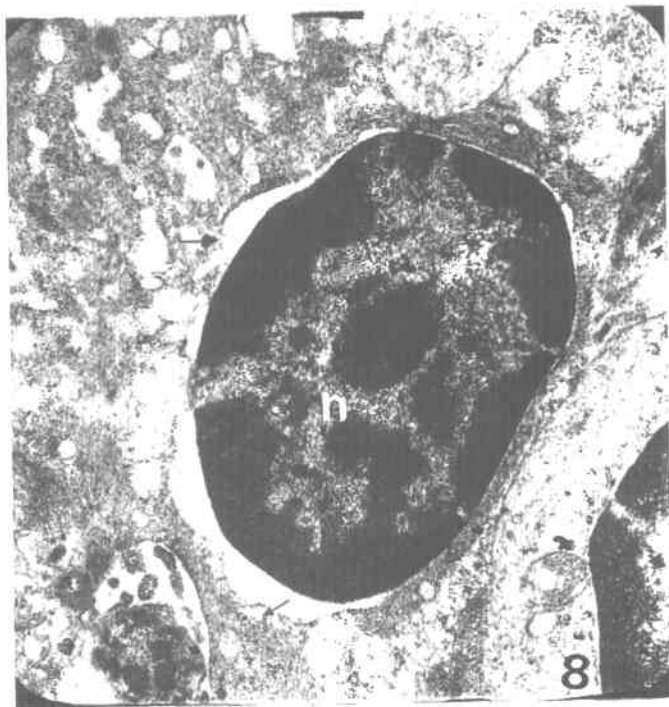
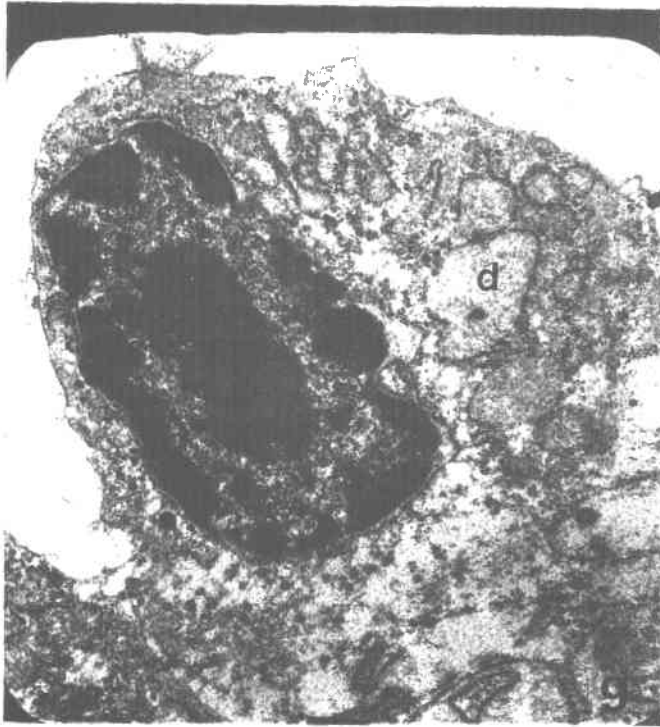
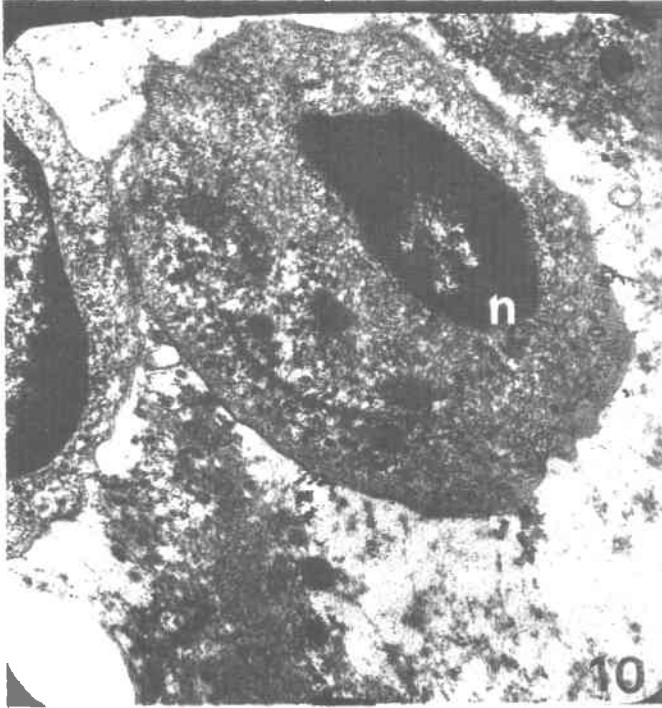


Fig. (8) : An electron micrograph of the spleen of a senile mouse showing a small lymphocyte with rounded nucleus (n) and degenerated cytoplasm. Notice the disrupted plasmalemma (↑).  
(Uranyl acetate & lead citrate; x 10,000)

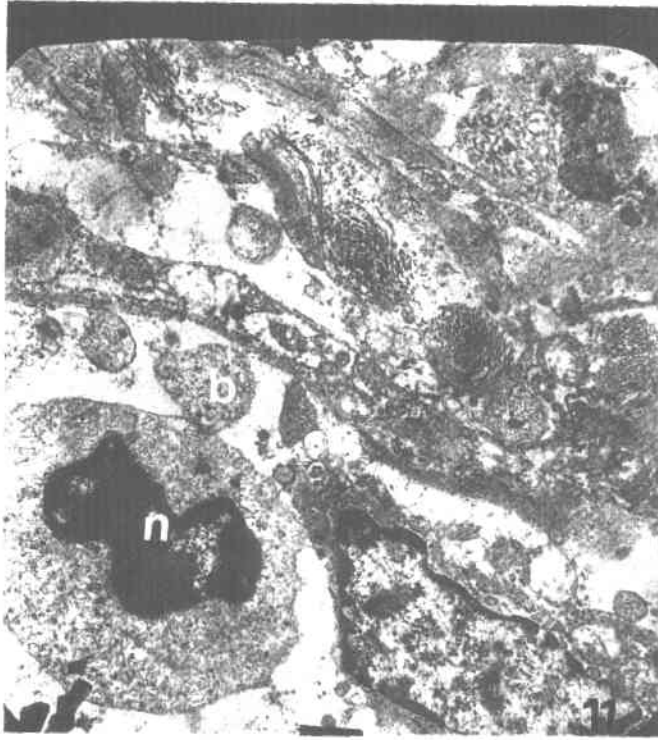


**Fig. (9) :** An electron micrograph of the spleen of a senile mouse showing the cytoplasm of a large lymphocyte with multiple vacuolar degeneration (d).

(Uranyl acetate & lead citrate; x 10,000)



**Fig. (10)** : An electron micrograph of the spleen of a senile mouse showing a lymphocyte with pyknotic nucleus (n). The cytoplasm reveals scattered lysosomes (l).  
(Uranyl acetate & lead citrate; x 10.000)



**Fig. (11) :** An electron micrograph of the spleen of a senile mouse demonstrating lymphocyte with pyknotic nucleus (n) and large cytoplasmic bleb (b).

(Uranyl acetate & lead citrate; x 6000)

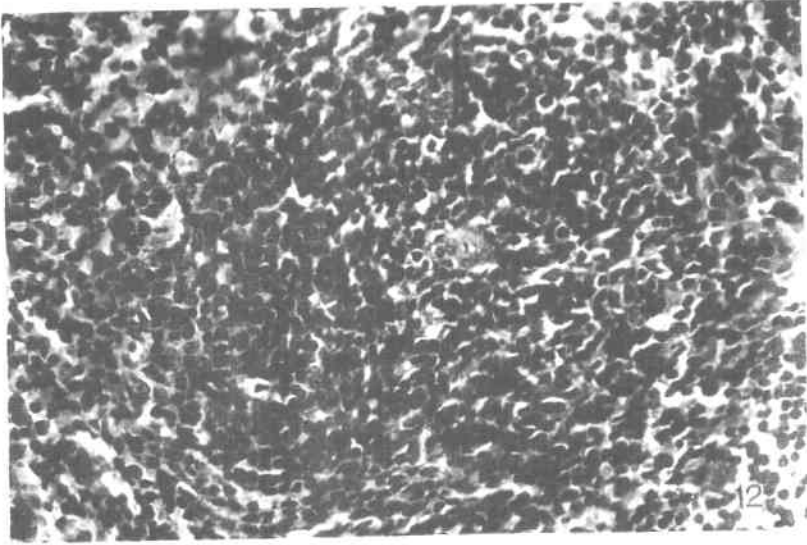


Fig. (12) : A photomicrograph of section of the spleen of an adult mouse which received interferon. Notice the prominent white pulp and loss of demarcation between the zones.

(Hx. & E.; x400)

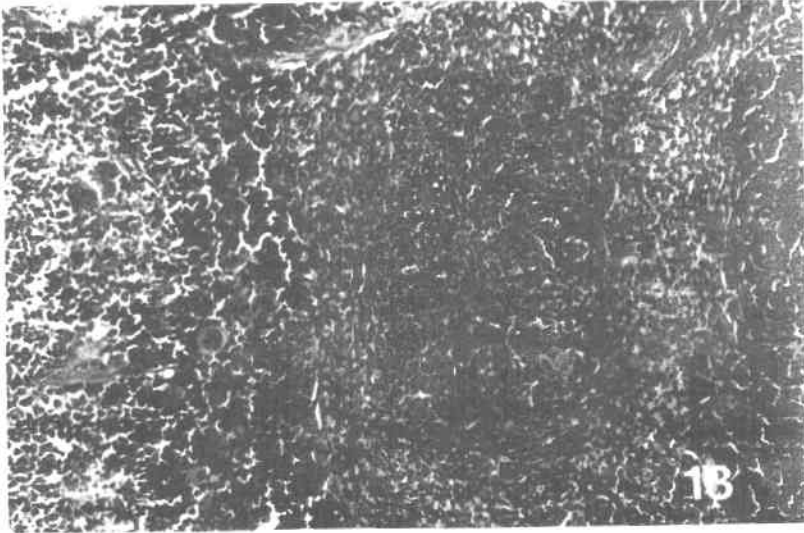


Fig. (13) : A photomicrograph of a section of the spleen of an adult mouse which received interferon showing the variation in the condensation of lymphocytes. Notice the regressed red pulp (↑).

(Hx. & E.; x 200)

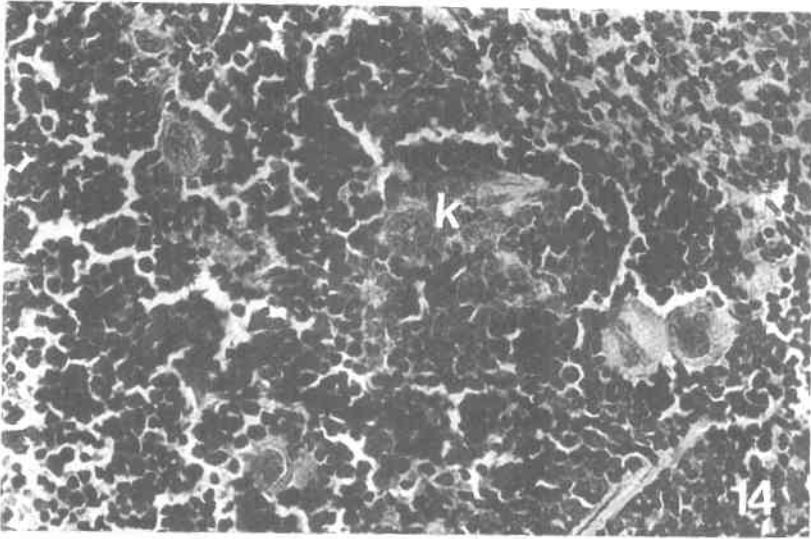
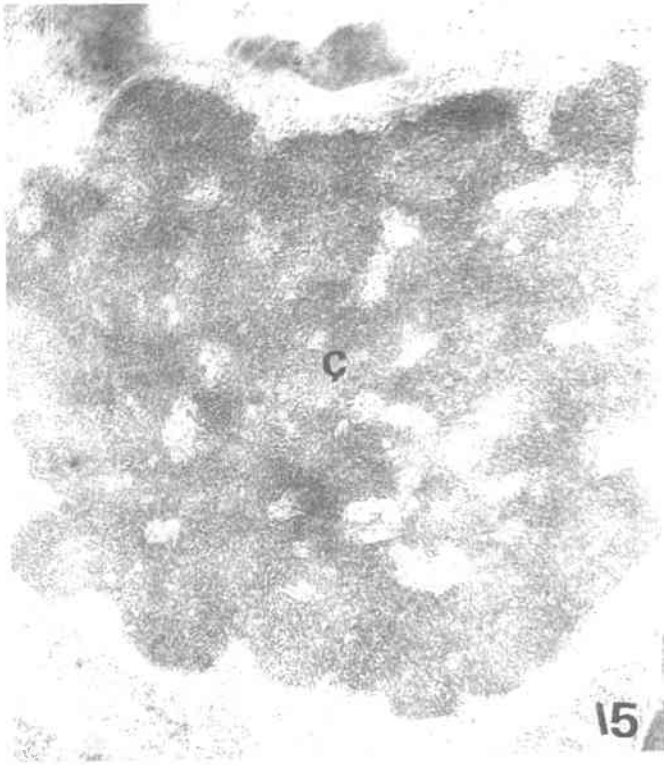


Fig. (14) : A photomicrograph of a section of the spleen of an adult mouse which received interferon showing megakaryocytes (k). (Hx. & E.; x 400)



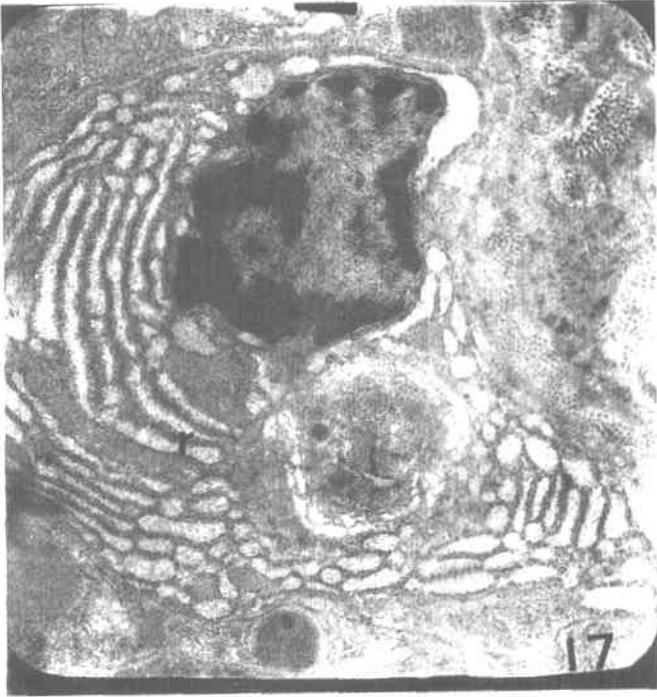
**Fig. (15) :** An electron micrograph of the spleen of an adult mouse which received interferon showing an enlarged, homogeneous dispersed chromatin (c).

(Uranyl acetate & lead citrate; x 22,000)

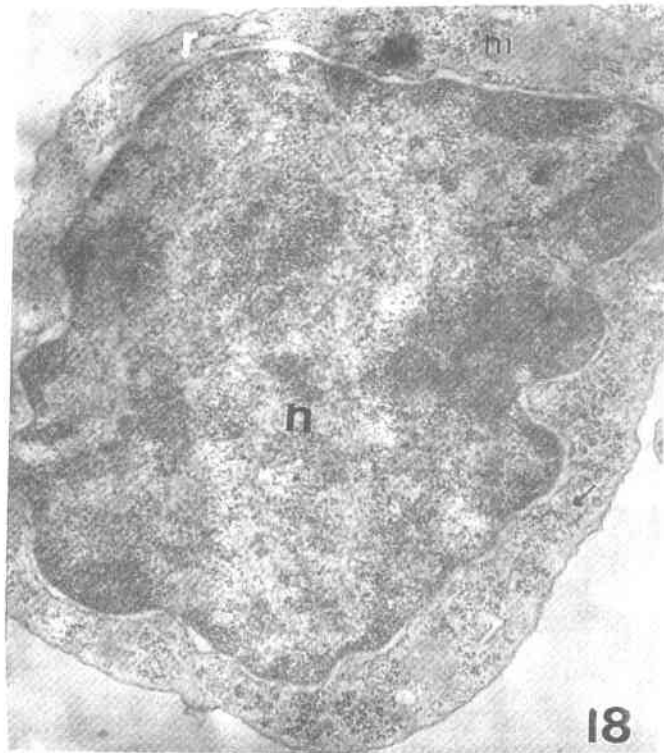


**Fig. (16) :** An electron micrograph of the spleen of an adult mouse which received interferon showing loss of nuclear membrane ( $\uparrow$ ). Notice the dense multiple chromatin clumps (c).  
(Uranyl acetate & lead citrate; x 10,000)





**Fig. (17)** : An electron micrograph of the spleen of an adult mouse which received interferon demonstrating marked proliferation of rough endoplasmic reticulum (r).  
(Uranyl acetate & lead citrate; x 3000)



**Fig. (18)** : An electron micrograph of the spleen of an adult mouse which received interferon showing a lymphocyte with euchromatic nucleus (n). The cytoplasm shows dilated cisterns of rough endoplasmic reticulum (r) mitochondria (m) and numerous polyribosomes (↑).  
(Uranyl acetate & lead citrate; x 17,000)

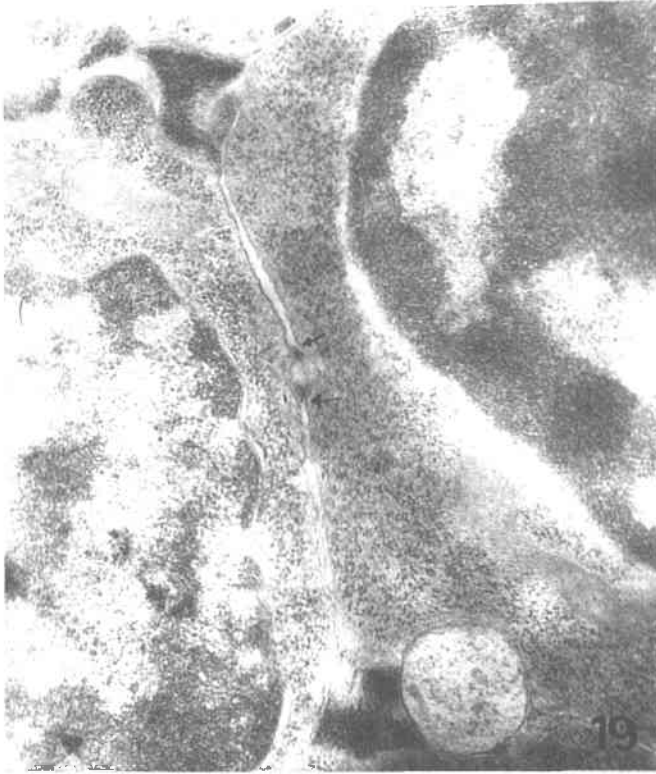
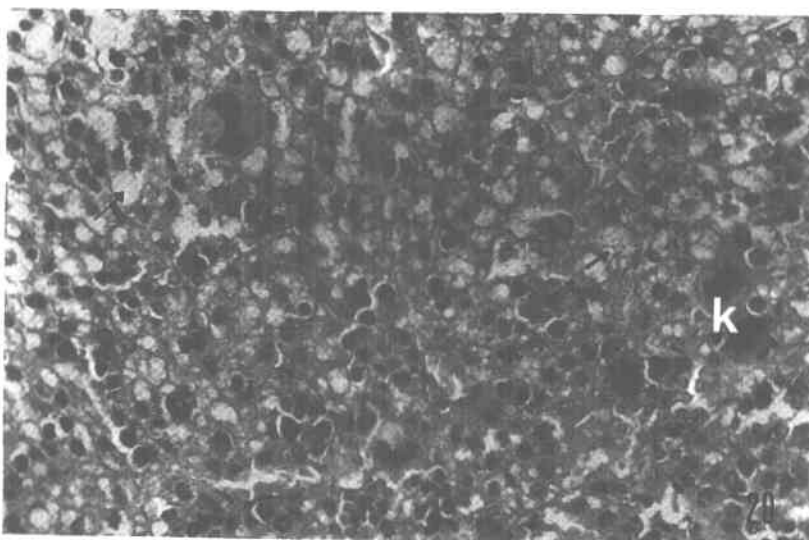
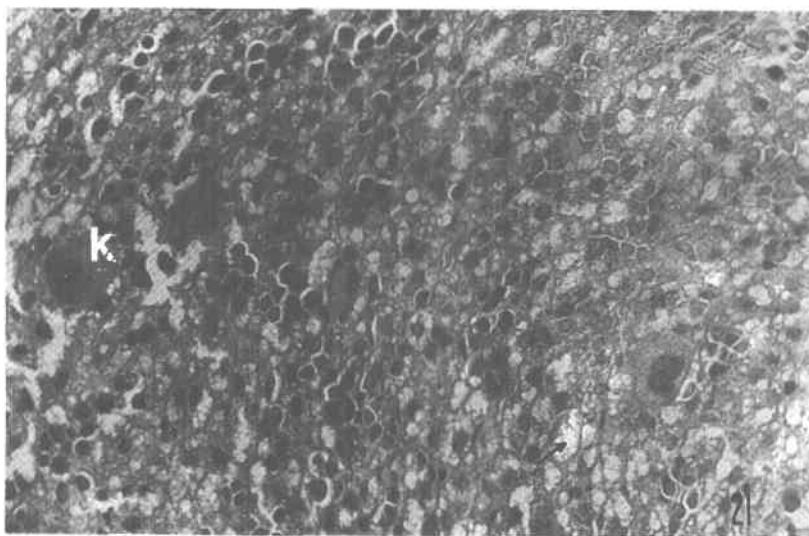


Fig. (19) : An electron micrograph of the spleen of an adult mouse which received interferon illustrating opposed adherent plasmic membranes at localized sites (1).

(Uranyl acetate & lead citrate; x 22,000)



**Fig. (20) :** A photomicrograph of section of the spleen of a senile mouse which received interferon showing distorted splenic architecture and depletion of lymphocytes in white pulp ( $\uparrow$ ). Notice the megakaryocytes (k).  
(Hx. & E.; x 400)



**Fig. (21) :** A photomicrograph of a section of the spleen of a senile mouse which received interferon demonstrating multiple vacuolation in the red pulp ( $\uparrow$ ). Notice the megakaryocytes (k).

(Hx. & E.; x 400)

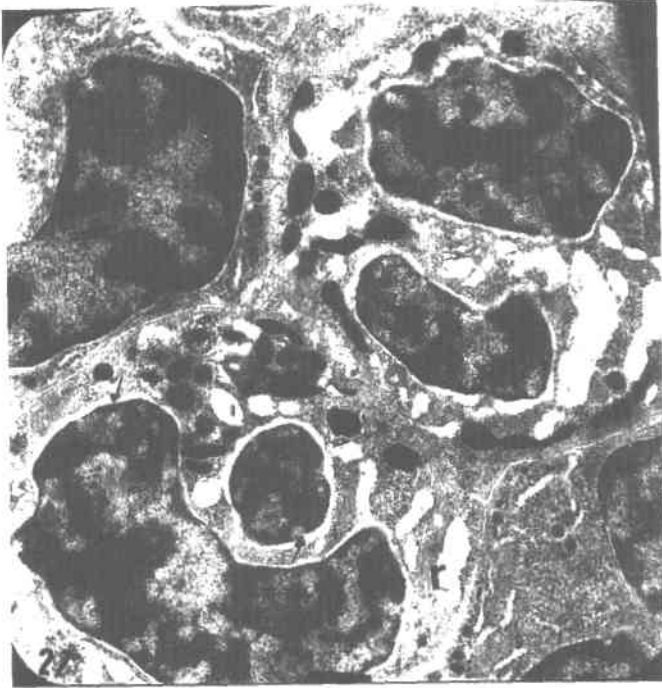


Fig. (22) : An electron micrograph of the spleen of a senile mouse which received interferon demonstrating lymphocytes with multiple dilated cisterns of rough endoplasmic reticulum (r) and detached ribosomes. Notice the widening of perinuclear space (↑).  
(Uranyl acetate & lead citrate; x 3000)

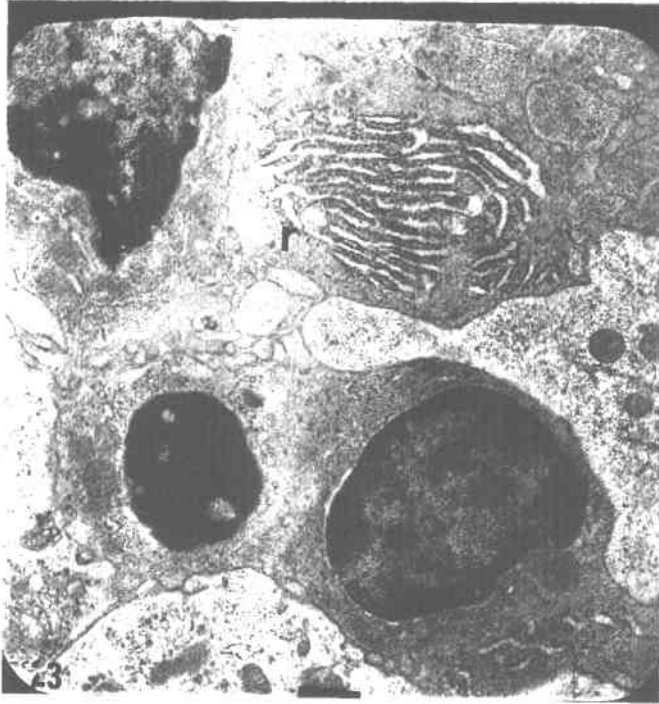


Fig. (23) : An electron micrograph of the spleen of a senile mouse which received interferon showing proliferation of the rough endoplasmic reticulum (r).

(Uranyl acetate & lead citrate; x 3000)

## DISCUSSION

The present study clarified that administration of interferon alpha has provoked profound alterations in the splenic architecture. It enhanced the proliferation of lymphoid tissue essentially required to allow interferon to exert its immunomodulatory effects in adults. However, aging seems to alter the response of the spleen.

Following administration of interferon, the spleen of adult mice exhibited impressive prominent white pulps. Moreover, the present work revealed that the distinct zones of white pulp, namely, the PALS, the lymphatic follicles and the marginal zone were amalgamated together forming a confluent mass of condensed lymphocytes. However, the degree of condensation of lymphocytes was variable. The extensive amalgamation and accumulation of lymphocytes could be attributed to the ability of interferon to induce traffic of lymphocytes within the spleen. Moreover, interferon could enhance pooling of lymphocytes from bone marrow and facilitate their subsequent accumulation in the other compartments as the spleen or lymph nodes (Salazar et al., 1996). The characteristic finding of the present study was the apparent regression of the red pulp as clusters of small lymphocytes entirely encroached on the red pulp. The lymphocytes had dark basophilic nuclei, narrow rim of cytoplasm and were more or less similar to those seen in the (PALS) and lymphatic follicles. Interferons could relocalize cells from white pulp to red pulp region or enhance the traffic along the splenic red pulp. Such migratory pattern might localize lymphocytes in a special order to receive additional activation signals or might play a role in lymphocytic proliferation (Salazar et al., 1996). Interferons have been showing to up regulate adhesion molecules important for different phases of lymphocyte migration from the blood via endothelial venules or through the lymphoid organs (Pabst & Westermann, 1994).

The present work further demonstrated that the majority of lymphocytes exhibited signs of activation and proliferation. Ultrastructural examination clarified that the nuclei had either enlarged dispersed homogenous chromatin or manifested loss of nuclear membrane, nucleolus and the chromatin was arranged as dense blocks on either sides of the cell. The morphological appearance of chromatin encountered represent the prophase stage of mitosis (Junqueira & Carneiro, 2003). In addition, the cytoplasm exhibited striking proliferation of the rough endoplasmic reticulum which occupied the entire of the cytoplasm. The rough endoplasmic reticulum was concentrically arranged and consisted of regular cisterns of uniform caliber a part from few dilated cisterns. The expanded uniform proliferation of the rough

endoplasmic reticulum is a prominent morphological sign of lymphocytic activation (Singh et al., 1998). Structurally, these cells bear the features of plasma cells indicating that alpha interferon could initiate a synchronized terminal differentiation of lymphocytes into plasma cells. In vitro-studies indicated that interferon exclusively might have a proliferative enhancing effects on B cells. However, it was not clear if secondary mediators were participating in plasma blast formation (Steinger & Van der Meide, 1993). The activated B lymphocytes might achieve a faster and more coordinated immune responses which could be a part of the mechanism that allows interferon to achieve its immune modulator effects. (Bosio et al., 2001).

Although lymphocytes could be activated by interferon therapy, recently a group of natural interferon producing cells were recognized normally in the T cell zone of the lymph nodes and in the marginal zones of the spleen. Ultrastructurally, the natural interferon producing cells or the so called plasmacytoid dendritic cells display the ultrastructural characteristics resembling those of human plasmacytoid cells and could produce interferons after viral stimulation (Martin et al., 2002). It is postulated that interferon therapy initiates signals that are transduced via cell to cell interactions, a possible intermediate in the signal transduction pathway is the natural interferon producing cells which possess interferon receptors on their surface. Interferon therapy might help maturation and differentiation of natural interferon producing cells (Bosio et al., 2001). Moreover, it is hypothesized that the ability of natural interferon producing cells to respond to exogenous interferon is essential for generation of fully activated dendritic cells (Montoya et al., 2002).

The present work clarified another form of activated lymphocytes. The cells beared partially euchromatic nuclei, and their cytoplasm contained few dilated cisterns of rough endoplasmic reticulum, scattered mitochondria and numerous free ribosomes. These are probably activated T lymphocytes (Junqueira & Carneiro, 2003). The activation of various types of T lymphocytes following interferon alpha has been reported in previous experimental studies. Immunohistologic and flow cytometric analysis showed marked infiltration of activated T lymphocytes (CD8) in wild type tumors of mice inoculated with interferon alpha expression cells, suggesting that interferon alpha can induce cytotoxic T lymphocytes and elicit long lasting tumor immunity (Eguchi et al., 2003). Moreover, administration of interferon I mainly the beta subtype significantly increased the proportion of natural killer T lymphocyte (NKT) as well as natural killer cells (NK) in the spleen of rat (Suzuki et al., 2002).

The interferon activated lymphocytes particularly natural killer cells (NK) can produce a variety of growth factors for hematopoiesis and exert significant



hematopoietic growth promoting effects (Murphey et al., 1992). The hematopoietic effect of natural killer cells could explain the presence of numerous megakaryocytes noticed in the present study in the red and white pulps of adult spleen following interferon administration. The present work further clarified a peculiar phenomena as it revealed that the plasmic membranes of some lymphocytes were opposed and adherent at localized sites. Such adhesion of lymphocytes might allow cooperation or might even assist in the movement of stimulating and differentiating factors between the adjacent lymphocytes to enhance the immune responses (Han et al., 2003).

The mitotic morphological changes noticed in the present study is a visible manifestation of cell division, but other processes play a fundamental role in the cell multiplication among these is the phase where DNA replicates during the interphase. The interphase is divided mainly into  $G_1$  phase (presynthesis), S phase (DNA synthesis) and  $G_2$  phase (DNA duplication) (Junqueira & Carneiro, 2003). The present study demonstrated by image analysis of Feulgan blue stained sections that the majority of lymphocytes of adult spleen were at  $G_1$  phase and S phase of cell cycle with a medium proliferative index. On the other hand, few lymphocytes were at  $G_0$  phase and to a lesser extent at  $G_2$  phase or aneuploid phase of cycle. Administration of interferon to adult mice induced prominent alterations in the cell cycle. Interferon significantly increased the percentage of lymphocytes at S phase with a high proliferative index. The lymphocytes at S phase represented the majority of the cells. Interferon, as well, significantly increased the lymphocytes at  $G_2$  phase and aneuploid phases. These changes were associated with simultaneous significant reduction in the percentage of lymphocytes at  $G_1$  phase. Moreover, no lymphocytes could be observed at  $G_0$  phase of the cell cycle. These results suggest that following interferon the majority of lymphocytes were in the active phase of cell cycle and might indicate a close relationship between the proliferative immune enhancing action of interferon and the cell cycle. The enhancing effects of interferon on the lymphocytes at S and  $G_2$  phases are contrary to its effects on the hepatocytes. It was reported that interferons decrease the number of hepatocytes at S and  $G_2$  phases of the cell cycle while they increase the number of hepatocytes in  $G_0$  and  $G_1$  phases. Interferon suppresses the proliferation of liver cell by inducing cell arrest and apoptosis mainly at the S and  $G_2$  active phases of the cell cycle (Kim et al., 2000).

The present work further revealed that the DNA content of lymphocytes at S phase  $G_2$  phase and aneuploid phase was significantly increased. Although the lymphocytes at  $G_1$  phase were significantly reduced in number yet their DNA content was also significantly increased. Again, these results were contrary to the reported

effects of interferon on hepatocytes. It has been shown that interferon suppresses hepatic DNA synthesis in mice. The inhibitory effects were attributed to suppression of cyclic adenosine monophosphate and ornithine decarboxylase enzyme activities in the hepatic cells (Theocharis et al., 1997).

Regarding senile mice, the present work clarified that aging induced eminent morphological changes in the spleen. The white pulp manifested marked regression with loss of the distinct demarcation of the different zones. Moreover, the present study illustrated marked depletion of lymphocytes in the white and red pulps. PALS is believed to be a T lymphocyte dependant zone while the marginal zones and lymphatic follicles are mainly B lymphocyte regions (Steinger & Van der Meide, 1993). Pronounced depletion was noticed in the present study in the lymphatic follicles and the marginal zone. PALS exhibited sparse lymphocytes that surrounded arterioles in an irregular pattern. The generalized depletion of lymphocytes encountered in the present work is suggestive of depletion of both B and T lymphocytes. Aging is believed to be associated with reduction in B cell population and reduction in all subtypes of T cells including CD<sub>4</sub> and CD<sub>8</sub> (Watanabe et al., 2001; Woods et al., 2003). The existing T cells are less capable of promoting B cell differentiation (Howel et al., 2003). However, the ability of the existing B lymphocytes to respond to antigens and to differentiate into high affinity antibody secreting cells appeared to be intact (Daiely et al., 2001).

Ultrastructural studies of the spleen of senile mice showed that the small lymphocytes had intact nuclei but the cytoplasm was degenerated. Moreover, the plasmalemma was disrupted and the lymphocytes lacked the distinct cellular boundaries. The damage of the cell membrane could be attributed to age associated release of free radicals. Membranes damaged by radicals usually become more rigid and exhibit decrease permeability. These changes could lead to reduced function and in fact might result in disruption of cell membrane (El Sökkary et al., 2003).

Large lymphocytes manifested marked vacuolar cytoplasmic degeneration and ill-defined cytoplasmic organelles. Moreover, some lymphocytes exhibited in addition to cytoplasmic degeneration numerous pyknotic nuclei. Large cytoplasmic blebs enclosed by plasmic membranes were noticed protruding from the cell surface. Such cells are presenting signs of apoptosis. Enhanced apoptosis in aging lymphocytes was recently attributed to oxidative stress (Schindowski et al., 2001). Apoptotic cells alter the immune responses and reduce the overall activity of immune competent cells. Moreover, it might be the cause of the decline in humoral immunity associated with aging (Eaton-Bassirie et al., 2000).

Image analysis of the Feulgen blue stained sections revealed that the majority of lymphocytes were at  $G_1$  phase and S phase of cell cycle with a medium proliferative index. Both phases did not exhibit significant variation in their percentage of cells nor in their DNA content than adult mice. On the other hand the percentage of lymphocytes at  $G_0$  phase was significantly increased simultaneously with significant decrease in their DNA content. The alterations encountered in the  $G_0$  phase in the senile cell cycle is suggestive of increase tendency of arrest of cell division. This could be probably attributed to increase in DNA damage and mutations that occur with age, associated with reduced ability to repair the damaged DNA (Cabelof et al., 2002). Furthermore, the decrease in the DNA content could be attributed to the increase incidence of anti DNA auto antibodies which localize within B cell follicles (Eaton - Bassirie et al., 2000).

Following administration of interferon to senile mice the spleen exhibited distorted and exhausted architecture. Multiple areas of depletion were encountered in the white pulp together with multiple vacuolation in the red pulp. Numerous lymphocytes were diffusely scattered without discrimination. In spite of the encountered depletion, the existing lymphocytes did not reveal by light or electron microscopic examination evident signs of apoptosis. The lack of apparent cellular necrosis in the small and large lymphocytes in spleen of senile animals which received interferon might suggest that the depleted lymphocytes left the spleen in response to interferon administration. It could be speculated that these cells were mobilized from spleen to peripheral areas and that such mobilization is a part of mechanism that might allow interferon to be effective in senile mice (Bosio et al., 2001). The inability of lymphocytes to replenish the peripheral compartments in aged mice is attributed to the decline in lymphopoiesis with increasing age and the dramatic decline of the pre B cells (Johnson et al., 2002). The present work revealed that the lymphocytes exhibited dilated cisterns of rough endoplasmic reticulum with detached ribosome. Some lymphocytes exhibited proliferation of the rough endoplasmic reticulum. Image analysis of the Feulgen blue stained sections of the spleen of senile mice which received interferon revealed significant increase in population of lymphocytes at S and  $G_2$  phases of the cell cycle. On the other hand lymphocytes at  $G_1$  phase were significantly reduced than senile spleen. No lymphocytes could be detected at  $G_0$  phase. These changes were associated with significant increase in DNA content of lymphocytes at  $G_1$  and S phases.

Aging usually increases the incidence of arrest of lymphocytes and decreases proliferation of lymphocytes. The lack of lymphocytic proliferation in aged mice

could be due to several factors including the diminished lymphoproliferative effects of neuropeptide Y with aging (Medina et al., 2000). It could also be attributed to the less supportive function of aged bone marrow to the proliferation of splenic B cells to plasma cells (Han et al., 2003). Reduced proliferative capacity of both T and B cells could be a result of telomere shortening and end-to-end chromosomal fusion with aging (Blasco, 2002). However, in the present study it seemed that interferon was capable of increasing the proliferative index of existing lymphocytes in aged mice and enhancing the presence of numerous lymphocytes in the active phases of cell cycles.

In conclusion, interferon increased the activation and proliferation of lymphocytes in adult mice. Aged mice exhibited marked degenerative changes. However, on administration of interferon, though the spleen showed distorted architecture and areas of depletion, yet existing lymphocytes manifested signs of proliferation that might allow interferon to achieve its immuno modulatory functions.

#### SUMMARY

Interferon has proved to be effective in treatment of chronic viral hepatitis and a wide variety of autoimmune diseases. The present study aimed to investigate the effects of interferon alpha on the lymphoid tissue of the spleen of adult and aged mice. Following subcutaneous administration of interferon to adult mice for three months, the spleen exhibited prominent white pulp encroaching on the regressed red pulp. The zones of the white pulp were amalgamated together with indistinct boundaries forming a confluent mass of lymphocytes. Ultrastructural examination clarified that the lymphocytes manifested signs of mitosis and marked proliferation of the rough endoplasmic reticulum. Other lymphocytes had euchromatic nuclei, dilated cisterns of rough endoplasmic reticulum, numerous mitochondria and scattered ribosomes. DNA analysis revealed significant increase in percentage of lymphocyte at S and G<sub>2</sub> phases of cell cycle associated with significant decrease in percentage of cells at G<sub>1</sub> phase. DNA content was significantly increased.

The spleen of senile mice presented regressed white pulp. Marked depletion of lymphocytes was noticed in both red and white pulps. Ultrastructural studies revealed vacuolar cytoplasmic degeneration, pyknotic nuclei and cytoplasmic blebs in the lymphocytes. Although the majority of lymphocytes were at G<sub>1</sub> and S phases of cell cycle yet there was significant increase in percentage of G<sub>0</sub> cells with significant decrease in its DNA content.

Administration of interferon to senile mice for three months revealed distorted architecture. The spleen showed areas of depletion in white pulp and vacuolation in red pulp. However, the existing lymphocytes did not exhibit signs of apoptosis or pyknosis. Ultrastructural examination revealed dilated cisterns of rough endoplasmic reticulum. Other lymphocytes showed proliferation of the rough endoplasmic reticulum. The majority of lymphocytes were at S and G<sub>2</sub> phase and were significantly increased than in senile spleen. The percentage of lymphocytes at G<sub>1</sub> phase was significantly decreased. The mean value of DNA content of G<sub>1</sub> and S phases was significantly increased.

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تأثيرات انترفيرون ألفا على النسيج الليمفاوى  
فى طحال الجرذان البالغة والمسنة

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أثبت عقار الانترفيرون فاعلية فى علاج الالتهاب الكبدى الفيروسى المزمن وأمراض المناعة الذاتية . استهدف هذا البحث دراسة تأثير عقار انترفيرون ألفا على النسيج الليمفاوى فى طحال الجرذان البالغة والمسنة .

عقب إعطاء الانترفيرون تحت الجلد لمدة ثلاثة شهور للفئران البالغة . أظهر الطحال أن اللب الأبيض أكثر وضوحاً وانتشاراً وطفى على اللب الأحمر الضامر . أما مناطق اللب الأبيض فقد اندمجت بدون حدود واضحة . أوضح الفحص المجهرى أن الخلايا الليمفاوية بها انقسام ميتوزى وشبكة اندوبلازمية خشنة متكاثرة ، واحتوت خلايا ليمفاوية أخرى على أنوية ذات كروماتين سوى وشبكة أندوبلازمية متسعة الطبقات ومتكندرات متعددة وأجسام ريبوزومية متناثرة . وتحليل الحامض النووى (DNA) ظهرت زيادة نسبية فى نسبة الخلايا الليمفاوية فى مرحلة ( $G_2, S$ ) وانخفاض نسبي فى نسبة الخلايا فى مرحلة ( $G_1$ ) كما ازدادت كمية الحامض النووى نسبياً .

أظهر طحال الفأر المسن ضمور فى اللب الأبيض وظهرت مناطق مفرغة فى اللب الأبيض والأحمر . وأوضح الفحص المجهرى وجود فراغات سيتوبلازمية ونوايا متغلظة ، وفقاعات أو نقاط سيتوبلازمية فى الخلايا الليمفاوية . وبالرغم من أن معظم الخلايا الليمفاوية كانت فى مرحلتى ( $G_1, S$ ) كانت هناك زيادة نسبية فى نسبة الخلايا فى مرحلة ( $G_0$ ) وقل بها الحامض النووى (DNA) .

عقب إعطاء عقار الانتريفيرون للجرذان المسنة تبين أن الهيكل العام للطحال مضمحل وأظهر الطحال أماكن مفرغة في اللب الأحمر ومع ذلك لم تظهر الخلايا الليمفاوية أى علامات تفلظ أو ضمور وأظهر الفحص المجهرى اتساعاً فى طبقات الشبكة الأندوبلازمية وأوضحت بعض الخلايا الليمفاوية تكاثر فى الشبكة . وكانت معظم الخلايا الليمفاوية فى مرحلتى (G<sub>2</sub>, S) وأظهرت زيادة نسبية مقارنة بالجرذان المسنة كما قلت نسبة الخلايا فى مرحلة (G<sub>1</sub>) وازدادت كمية الحامض النووى (DNA) نسبياً فى مرحلتى (G<sub>1</sub>, S) .

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