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Morphology

Ganglioside changes in the spinal cord of Lewis rats with chronic relapsing experimental allergic encephalomyelitis

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Chronic relapsing experimental allergic encephalomyelitis was induced in Lewis rats with purified guinea-pig myelin and complete Freund's adjuvant. Rats were sacrified at different phases of the disease (just before the onset of clinical signs, during the first clinical episode of EAE and during the first recovery). Gangliosides were extracted from the spinal cord, analysed after purification by two-dimension chromatography and quantified densitometrically. An increase of GM1 and a decrease of GT1b were observed during the development of the EAE.

Key words: gangliosides, spinal cord, chronic relapsing experimental allergic encephalomyelitis, Lewis rats, demyelination.

Introduction

Experimental allergic encephalomyelitis (EAE) is a well established animal model for the human demyelinating disease, particularly multiple sclerosis (MS). EAE may have either an acute or a chronic relapsing course, but each form produces the same neurological signs, namely tail paralysis and limb ataxia, weakness and paralysis. Acute EAE is a monophasic disease, while chronic relapsing EAE (CR-EAE) has a relapsing remitting course resembling MS. Several data from both in vitro and in vivo experiments suggested that a role of gangliosides in the demyelinating process should be recognized [10].

Gangliosides are sialic-acid-containing glycosphingolipids found in high concentration in brain. They are true constituents of the myelin membrane, comprising 0,3—0,7 % of the total lipid (dry weight) [1].

To investigate the role of gangliosides in the pathogenesis of demyelinating diseases, in the present study the total concentration and the relative distribution of major spinal cord gangliosides (GM1, GD1a, GD1b and GT1b) of Lewis rats with CR-EAE, induced by inoculation with purified guinea-pig myelin and complete Freund's adjuvant were determined during the different phases of the disease. It was found an increase of GM1 and a decrease of GT1b during the development of the disease.

Material and Methods

Chronic relapsing experimental allergic encephalomyelitis was induced in Lewis rats (JC strains). Each batch of inoculum was prepared by homogenizing a mixture of 1 mg guinea-pig myelin, 0,75 ml 0,9 % saline, 0,75 ml complete Freund's adjuvant (Difco) and 100 μg *Mycobacterium tuberculosis* H37R (Difco). Rats, 7—12 weeks old, were injected intradermally with 0,1 ml inoculum into the two rear foot pads. Control rats were inoculated as above except that the inoculum did not contain guinea-pig myelin. The animals were weight and examined daily from the seventh day postinoculum (DPI) for clinical symptoms of EAE which included evidence of weakness, loss of tail tonicity, hindlimb paraparesis and paralysis, quadriparesis, quadriparalysis.

Animals were killed by decapitation at various stages of CR-EAE as follows: I group — preclinical stage (just before the onset of clinical signs — at 9 DPI); II group — first clinical episode of EAE (hindlimb paralysis — at 11—16 DPI); III group —

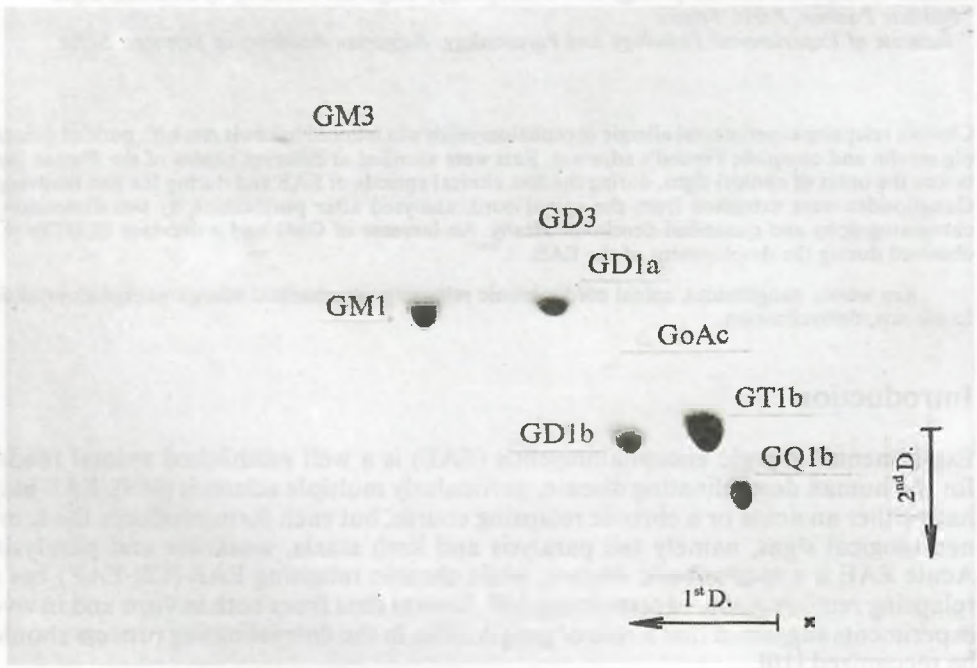

Fig. 1. Two-dimensional high-performance thin-layer chromatography of spinal cord of Lewis rats with CR-EAE — 43 DPI. The plate was developed with system solvents: 1st dimension — chloroform /methanol/ Aq. $CaCl_2$ (0,3 %) — 55/45/10; 2nd dimension — *n*-propanol /amoniaque conc./ water — 6/4/1

first recovery (no clinical signs — at 28—43 DPI); IV group — control animals. Spinal cords are quickly removed and weight. The total lipids were extracted from spinal cord (20,5 g wet weight) with 20 volumes of chloroform/methanol/ water (4:8:3 by volume). After centrifugation the clear supernatant was removed and the pellet subsequently re-extracted with 50 ml chloroform/methanol/ water (4:8:3) and 50 ml chloroform/methanol (2:1 by volume). The pooled extracts were dried by evaporation, redissolved in 40 ml chloroform:methanol (1:1 by volume), sonificated for 2 minutes and allowed to stand overnight at — 20°C. Than extracts was centrifuged and the clear supernatant, containing the total lipid extract, was dried by rotary evaporation.

Purification of gangliosides from the total lipid extract was performed according procedure of L a d i s h and G i l l a r d [4]. The gangliosides were analysed by two-dimensional thin-layer chromatography and quantified densitometrically (Fig. 1).

Results

The Lewis rats inoculated with guinea-pig myelin developed neurological signs by 11—14 DPI and recovered by 18—22 DPI. The second clinical episode commenced by 20—28 DPI and had been resolved by 23—48 DPI. The percentages of gangliosides

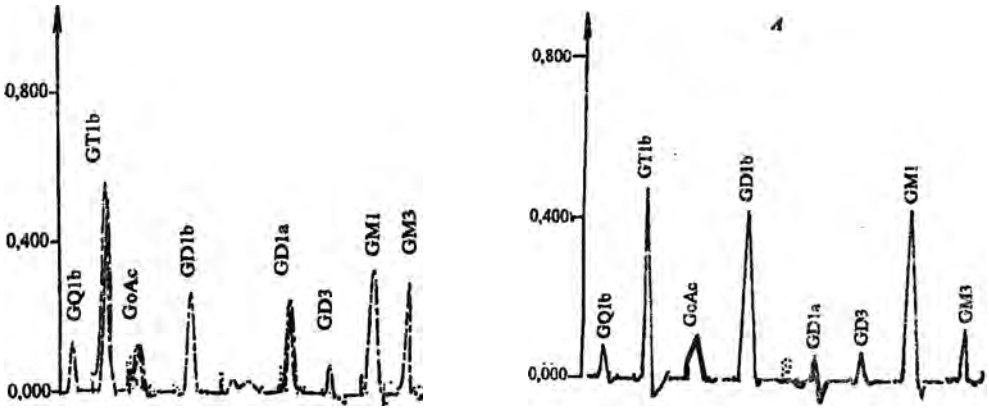

Fig. 2. Densitogram of the spinal cord gangliosides of Lewis rats (control group)

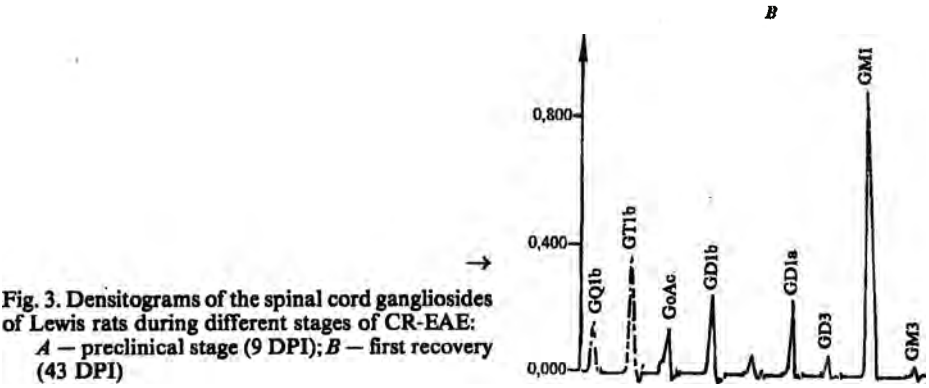

Fig. 3. Densitograms of the spinal cord gangliosides of Lewis rats during different stages of CR-EAE: *A* — preclinical stage (9 DPI); *B* — first recovery (43 DPI)

Table 1. Percentage distribution of gangliosides in Lewis rats spinal cord during different stages of CR-EAE

Ganglioside (rel. %)	I group	II group	III group	IV group
GT1b	43,2	30,9	19,2	51,6
GD1b	21,3	18,6	12,6	15,2
GD1a	16,5	17,2	8,5	7,4
GM1	19,0	33,3	59,7	25,8

in Lewis rats spinal cord during different stages of CR-EAE, were recalculated on the basis of the densitograms (Fig. 2, Fig. 3 and Table 1).

The data obtained demonstrate that during the development of CR-EAE the relative proportion of GM1 increases from 19,0% just before the onset of clinical signs to 59,7% during the first remission. There was 2,4 fold increase of this ganglioside in comparison with controls. The relative content of GT1b decreases from 43,2% (preclinical stage) to 19,2% (first recovery).

Discussion

The present study has revealed ganglioside changes in the spinal cord of Lewis rats with CR-EAE induced with purified guinea-pig myelin and complete Freund's adjuvant. It was found an increase of GM1 and a decrease of GT1b during the development of the disease (preclinical stage — first episode of EAE — first recovery). The ganglioside changes became more evident during the remission. The findings are in full concordance with our data concerning the ganglioside changes in the brain of Lewis rats with CR-EAE [13].

Chronic experimental allergic encephalomyelitis is an inflammatory demyelinating disease with clinical and pathological features resembling MS [5]. The histopathological changes in the central nervous system of the Lewis rats with CR-EAE have been described by P e n d e r et al. [7]. Our morphological examination of the Lewis rats with CR-EAE at light and electronmicroscopic levels (unpublished results) has also revealed inflammation and primary demyelination in the spinal cord during the first clinical episode. During the first remission, when the ganglioside changes were more evident, remyelination and demyelination were present, indicating ongoing disease activity.

It was suggested that the full picture of inflammatory demyelination is induced by a complex interaction of cellular and humoral immune reaction, and that in principle several different antigens of the central nervous system (CNS) can mediate autoimmune inflammation or demyelination respectively [6]. The main requirement for a myelin antigen as a target in antibody-mediated demyelination is its localization on the extracellular surface of myelin sheaths. Several such antigens have been identified including gangliosides GM1 [9]. They are some findings suggesting that GM1-antibodies impair myelin sheath. Antisera against a mixture of the major brain gangliosides GM1, GD1a, GD1b and GT1b or against GM1, injected into the lumbosacral subarachnoid space of normal rats, induced demyelination in spinal roots and spinal cord [9]. Antisera against GM1 exerted demyelination in well myelinated tissue culture [8].

On the other hand, it was suggested that GM1 and GT1b play a role in the myelin sheath formation [2, 3, 10, 11, 12].

In conclusion, the revealed changes of GM1 and GT1b in the spinal cord of Lewis rats during the development of CR-EAE (preclinical stage, first clinical episode and first remission) support the concept that gangliosides play a role in the pathogenesis of demyelination in CR-EAE.

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Rate constant of inhibition of cholinesterases by organophosphorus compounds: correlation between rate constant and toxicity

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A comprehensive analysis of the data on bimolecular rate constants of inhibition (BRCIs) of cholinesterases (acetylcholinesterase and cholinesterase) by organophosphorus compounds (OPCs), determined by recent methods described in the present manuscript, and LD_{50} values, reveals that there is an apparent proportionality between the values of the BRCIs of AChE and the degree of toxicity of OPCs. For strong neurotoxic agents the BRCIs exceed 10^6 M^{-1}. min^{-1}, whereas for the majority of less toxic OPCs this constant has lower values. We find it plausible to suggest the use of the bimolecular rate constant as a criterion for assessing the toxicity of OPCs, supplementing the routinely used toxicological techniques.

Key words: Inhibition constants of cholinesterases; Methods for determination of; Correlation with LD_{50}; Biological significance of.

Organophosphorus compounds have become a common element of humanity's household. The most widely distributed and easily accessible ones are those serving as pesticides. Another class of highly neurotoxic OPCs, namely the nerve gases intended for military use, has been accumulated throughout the world. Thus, OPCs have attracted a lot of attention and research efforts have been directed toward the creation of methods for estimation of their toxicity and the study of their mode of action.

The toxicity of dangerous drugs is assessed mainly by the LD_{50}-test, which is in use since 1927 [1]. During the last decade the correctness of the LD_{50} concept was reevaluated and a number of major drawbacks were pointed out: LD_{50} values obtained in animal experiments may be misleading if directly accepted for humans [1, 2]; a number of factors, such as species and strain, age, sex, diet etc., influence the LD_{50} values to an extent, which does not allow LD_{50} to be regarded as a biological constant [1]; we are now faced with important ethical considerations, which make animal experiments undesirable.

In this article we suggest the use of a biochemical constant, namely the BRCI, for the initial stages of the evaluation of the toxicity of organophosphorus compounds, prior to deployment of the traditional toxicological tests. For this purpose, we present

a concise review of methods for determination of the rate constant for the irreversible inhibition of AChE and ChE.

Methods for estimation of the bimolecular rate constant of inhibition

The bimolecular rate constant of the reaction of organophosphorus compounds with cholinesterases (AChE and ChE) has been studied intensively [see e.g. 3—14]. The experiments were carried out under conditions, where the reaction follows pseudo-first-order kinetics. The rate constant can be calculated according to Eq. (1), when the initial concentration of the strong irreversible inhibitor exceeds the initial concentration of the enzyme $[E]_o$ at least 20 fold [13. 15]:

(1) $$k_{II} = \frac{1}{t.[I]_o}.\ln(v_o/v_i),$$

where t is the time, during which the enzyme and the inhibitor have been in contact, k_{II} — the rate constant of the inhibition, $[I]_o$ — the intital (total) concentration of the inhibitor, v_o — the rate of the reaction in the absence of inhibitor, v_i — the rate of the reaction in the presence of inhibitor.

Under these conditions, the values obtained for k_{II} are numerically equal to the bimolecular rate constant [13].

The same method has been applied at an inhibitor to enzyme ratio lower than 20 [15]. However, under these conditions the calculation of k_{II} should be performed according to a different equation:

(2) $$k_{II} = \frac{1}{t([E]_o-[I]_o)}.\ln\left\{\frac{[I]_o([E]_o-[EI])}{[E]_o([I]_o-[EI])}\right\},$$

where $[EI]$ is the enzyme-inhibitor complex.

Another method is to preincubate the enzyme with several concentrations of the inhibitor, which allows the calculation of the partial inhibition (i), respectively — the estimation of the remaining enzyme activity (v_i/v_o). The inhibitor concentration and the time of preincubation have to be selected in such a way that the inhibition at the lowest inhibitor concentration (which in all cases should be 20 times higher than that of the enzyme) should be between 15 and 30%, and at the highest — from 70 up to 80% [16]. The rate constant k_{II} can be calculated with the help of Eqs. (3a) and (3b) from the value of $(-k_{II}t)$, which is equal to the slope of the straight line obtained on plotting "ln (100-i)" (which can be substituted by "$\ln(v_i/v_o)$") vs $[I]_o$ for a fixed preincubation time (in most cases 15—30 min, see e.g. [17—19]):

(3a) $$\ln(100-1) = 4{,}60517 - k_{II}t[I]_o,$$

(3b) $$\ln(v_i/v_o) = -k_{II}t[I]_o,$$

where 4,60517 = ln 100.

There are certain differences between the two methods, which have to be considered by the experimenter prior to making his choice. While the first method relies on the use of a single inhibitor concentration, the second one requires the use of several different concentrations. The first method is faster to perform, it still leaves some uncertainty whether the initial inhibitor and the initial enzyme concentrations have been properly selected to satisfy the requirement that the inhibitor concentration exceeds that of the enzyme by at least 20 times. On the other hand, with the second method one can be sure that the experimental conditions have been

chosen correctly if a straight line is obtained on plotting of ln(100-i) vs $[I]_o$ (see e.g. [20]).

The third method for calculating the value of the rate constant requires that the inhibitor concentration is far below the value of the dissociation constant. In this case the overall inhibition rate is determined by the bimolecular rate constant [21, 22]. This can be illustrated by the reaction between AChE (E) and an OPC (PX):

(4) $$E + PX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPX \xrightarrow{k_p} EP + X, \qquad k_{II}$$

where k_p is the phosphorylation constant, EP — the phosphorylated enzyme.

The dissociation constant (K_d) can be obtained from:

(5) $$K_d = k_{-1}/k_1.$$

Knowing the phosphorylation and dissociation constants, the bimolecular rate constant is calculated from:

(6) $$k_{II} = k_p/K_d.$$

This method is made less attractive by the fact that it requires the application of additional time consuming techniques, however, it allows the calculation of the values of further constants, characterizing the enzyme-inhibitor interactions.

Evaluation of the resistance of animals to organophosphorus compounds with the help of the rate constant

In order to characterize the apparent correlation between the rate constants of inhibition of cholinesterases by certain OPC and their LD_{50} values we summarized available published data [5-13, 17-19, 21, 23-27, 30, 32, 33, 37, 39, 41-46]. Since most of the authors work under compatible conditions we accept that a comparison of the reported data is viable.

A good example to illustrate this correlation are the two strongly toxic isomers of soman, namely $C_{(+)}P_{(-)}$- soman and $C_{(-)}P_{(-)}$-soman, characterized by low LD_{50}-values of 0,099 and 0,038 mg/kg (subcutaneous application, rat) respectively [23]. The rates of inhibition of electric eel AChE by these two soman isomers are $2{,}8.10^8$ and $1{,}8.10^8$ $M^{-1}.\ min^{-1}$ respectively [23]. The other two soman isomers are of much lower toxicity, which is reflected by their high LD_{50}-values exceeding 5 or 2 mg/kg (subcutaneous, rat) respectively, and by considerably lower $k_{II} \leq 5.10^3$ $M^{-1}.\ min^{-1}$ for both of them [23]. Similar results were reported by K e i j e r and W o l r i n g [24].

The values reported for the bimolecular rate constant of inhibition of electric eel AChE by certain potent neurotoxic agents, destined for military use [25] are: V_x — $2{,}54.10^7$ $M^{-1}.\ min^{-1}$; sarin — $1{,}55.10^7$ $M^{-1}.\ min^{-1}$; a racemic mixture of soman — $5{,}58.10^7$ $M^{-1}.\ min^{-1}$; $C_{(-)}$-soman — $4{,}08.10^7$ $M^{-1}.\ min^{-1}$; and $C_{(+)}$-soman — $6{,}32.10^7$ $M^{-1}.\ min^{-1}$. All of these compounds have correspondingly low LD_{50} -values. The inhibition of the same enzyme by the less toxic DFP (LD_{50} = 7,7-13,5 mg/kg, per os, rat) and paraoxon (LD_{50} = 3 mg/kg, per os, rat), is characterized by correspondingly low k_{II} -values of $9{,}48.10^3$ $M^{-1}.\ min^{-1}$ and $1{,}16.10^5$ $M^{-1}.\ min^{-1}$ [see e.g. 39].

Further analysis of the same set of data yielded support to the notion, that there is a clearly expressed correlation between the LD_{50}-values and k_{II} [see also 13, 17, 19,

26, 27]. A plot of LD_{50}-values vs k_{II} (Fig. 1) reveals that the strongly toxic nerve agents are characterized by inhibition constants exceeding 10^6 M^{-1}. min^{-1}, while less toxic compounds are characterized by inhibition constants with lower values. Therefore, we find it feasible to suggest the value of 10^5 M^{-1}. min^{-1} as a conventional criterion for the toxicity of organophosphorus compounds.

Furthermore the plot on Fig. 2 shows that the same correlation is found for compounds, known to be of low toxicity, and the only compound for which the k_{II} is higher than 10^6 M^{-1}. min^{-1} is tetraethyl pyrophosphate, previously used as a pesticide, however, because of its high toxicity its application has been limited [28].

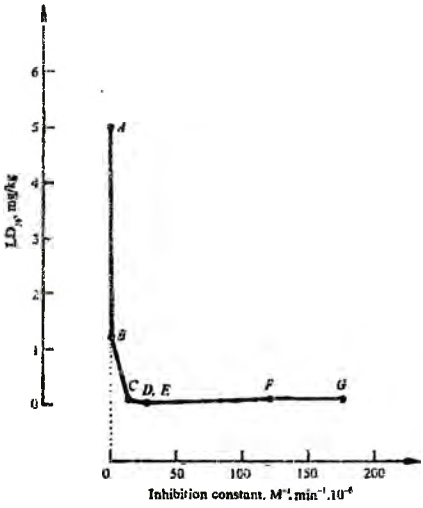

Fig. 1. Corellation between the toxicity and the inhibition constant of AChE inhibitors (subcutaneous application)
Capital letters on right of the points of the curve denote the organophosphorus compound studied: *A* — DFP; *B* — *S*-butyl-*o*-(4-nitrophenyl) methylthiophosphonate; *C* — $C_{(-)}$ $P_{(-)}$-soman (ref. 24); *D* — $C_{(-)}$ $P_{(-)}$ soman (ref. 23); *E* — $C_{(+)}$ $P_{(-)}$-soman (ref. 24); *F* — $C_{(+)}$ $P_{(-)}$-soman (ref. 23); *G* — Sarin. The vertical dotted line shows the position of k_{II}-value of 10^6 M^{-1}. min^{-1}

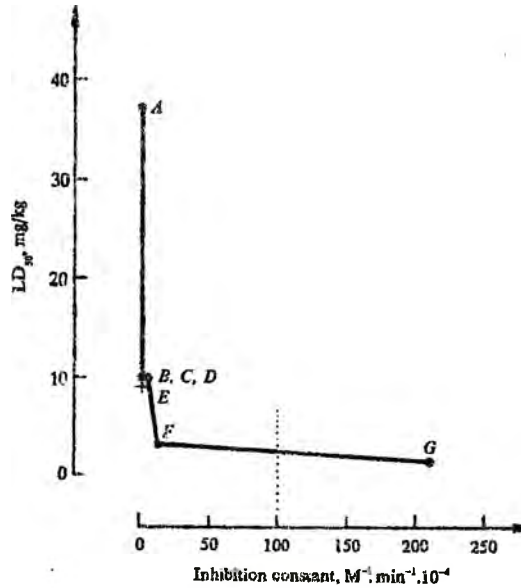

Fig. 2. Corellation between the toxicity and the inhibition constant of AChE inhibitors (per os intake)
Capital letters on right of the points of the curve denote the organophosphorus compound studied: *A* — Parathion-methyl; *B* — DFP (ref. 25); *C* — DFP (ref. 13); *D* — DFP (ref. 33); *E* — Parathion; *F* — Paraoxon; *G* — TEPP. The vertical dotted line shows the position of k_{II}-value of 10^6 M^{-1}. min^{-1}

We suggest that this criterion can be used to forecast the toxicity during early stages of investigations on OPCs. It can be expected that substances for which the k_{II} exceeds 10^6 M^{-1}. min^{-1} should be extremely toxic. If the expected high toxicity precludes the use of these substances in the household or in the agriculture, the tests for determining the LD_{50} of such compounds can be avoided, or in cases of great interest in a given OPC, one could at least lower considerably the number of animals used for LD_{50} tests.

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Developmental appearance of the nitric oxide synthase during postnatal gonadal development in the rat

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Nitric oxide is an intra- and intercellular messenger in physiological and pathophysiological processes in mammalian cells. NADPH-diaphorase staining histochemistry was applied to study the gonadal localization of nitric oxide synthase (NOS) in rat gonads. Ovaries and testes removed from neonatal, pubertal (15- and 30-day-old) and adult animals were investigated. In the testis the appearance of NOS-activity was closely correlated to active testosterone production. Its persistence in both foetal-type and adult-type Leydig cells (LC) at all stages was shown. In the ovary, the NOS-activity is located in theca cells, follicle cells, luteal and interstitial cells. The positive reaction was observed to be present in primary follicles at postnatal day 15. In adult ovary all steroidogenic cells were stained, the intensity of the reaction being most prominent in luteal and interstitial cells. These results suggest that NOS is expressed differently during gonadal development in the rat in close correlation to the steroid hormone production.

Key words: nitric oxide synthase, steroidogenic cells, gonads, development, rat.

Introduction

Recent investigations suggest that nitric oxide (NO) is of wide occurrence as a major messenger molecule regulating immune function and blood vessel dilatation and serving as a neurotransmitter in the brain and peripheral nervous system (S n y d e r, B r e d t, 1991; L o w e n s t e i n, S n y d e r, 1992). Moreover, NO is thought to represent an intra- and intercellular signal molecule in various mammalian tissues including brain, blood vessels, kidney, immune system etc (N a t h a n, 1992). Neuronal nitric oxide synthase (NOS) was recently shown to be a 150 kDa enzyme identical to NADPH-diaphorase (D a w s o n et al., 1991; H o p e et al., 1991). At present there is no specific method for the visualization of NOS using its catalytic properties. So far NO and its reaction products, NO_2 and NO_2^-, could not be used in a suitable procedure as could not the other final reaction product, citrulline. A suitable possibility offers the third final product, NADPH, i. e. using the NADPH-diaphorase activity of the enzyme (G o s s r a u, 1994). A one-to-one correlation between NOS-immunoreactivity and NADPH-diaphorase staining in some neurons was revealed indicating that NADPH-diaphorase histochemistry may be a good marker for cells containing NOS (D a w s o n et al., 1991; Y o u n g et al., 1992).

In this study we focused on the histochemical visualization of NOS in steroidogenic cells of developing rat gonads (Leydig cells, follicle cells, theca cells) using NADPH-diaphorase activity of the enzyme.

Material and methods

In this study Wistar rats at different developmental stages were used: neonates, pubertal (15 and 30-day-old) and adult animals. They were killed by decapitation. Gonads, ovaries and testes, were removed and tissue blocks were frozen in liquid nitrogen. Ten micron-thick cryostat tissue sections were fixed in 0,7 % formaldehyde in 0,1 M phosphate buffer, pH 7,6. The sections were then incubated for 1 h at 37 °C in a solution containing the fixative with addition of 1 mg/ml nitro-blue-tetrazolium (NBT) and 1 mg/ml NADPH. The sections were rinsed with distilled water and coverslipped with glycerol-gelatine. The sites of the NOS-activity were marked by formazan granules.

Results

I. Testis

In testicular sections at all developmental stages studied the NOS-activity was always present in the interstitium.

In neonates, the fetal-type LC appeared stained and the reaction product was localized in the cell body (Fig. 1).

In rats, the second proliferation of testosterone producing cells appeared between days 12 and 15 after birth and lasted from puberty to adulthood during maturation of adult testis and testicular functions (L o r d i n g, D e K r e t s e r, 1972). Histochemically

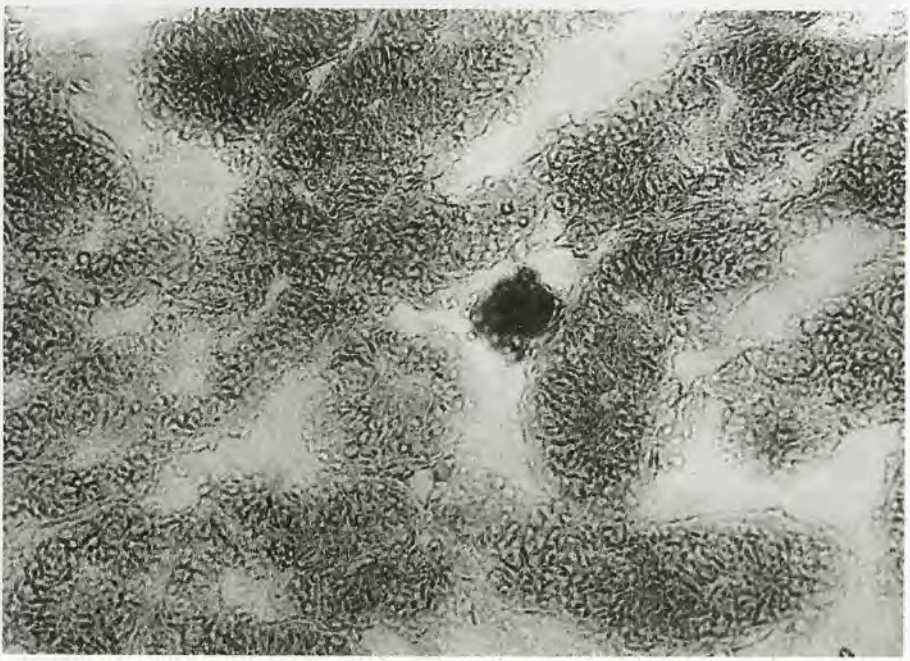

Fig. 1. Neonatal testis. A group of Leydig cells between seminiferous cords is strongly diaphorase-reactive. ×160

Fig. 2. 15-day old testis. A moderate NOS-activity in the second generation of Leydig cells is visible (→). ×160

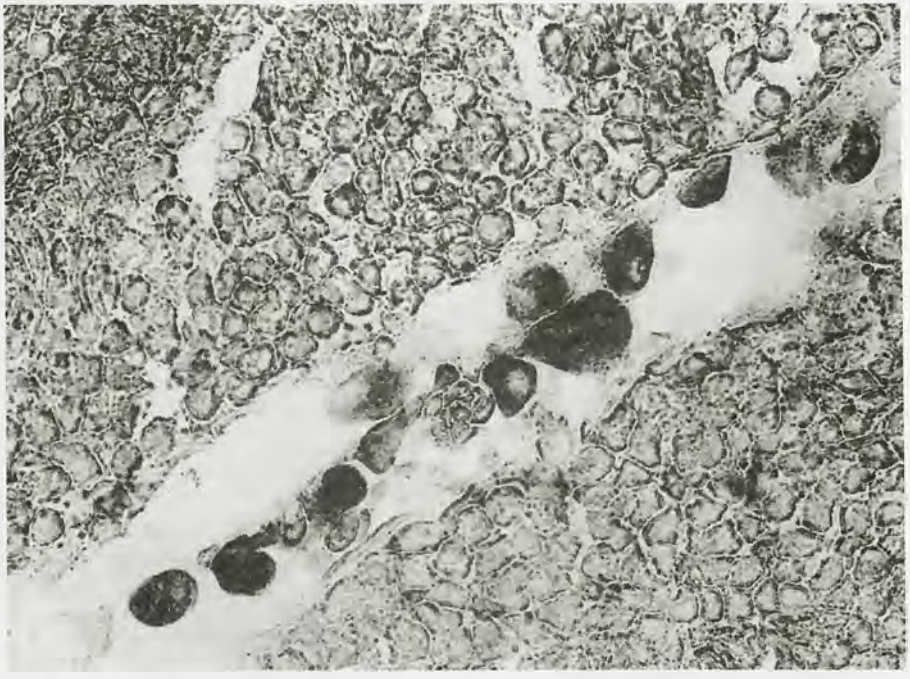

Fig. 3. Testis from an adult animal. Formasan granules in the cytoplasm of Leydig cells are observed pointing to a strong NOS activity. ×400

NOS-activity was also observed in the LC. Located between seminiferous tubules they contained formazan granules in their cytoplasm. Nuclei remained unreactive at all developmental stages under study.

In the interstitial tissue of the 15-day-old testis LC were faintly diaphorase stained (Fig. 2). Later, on day 30 post partum,a more intense reaction was visualised.

In adult testis a strong NOS-activity was present in well differentiated Leydig cells (Fig. 3). No staining was observed in seminiferous tubules.

II. Ovary

In the ovary, the principal sites of steroid hormone production are follicle cells, theca cells and luteal cells. The interstitial tissue is the main source of ovarian androgens.

In neonatal rat ovary, the oocytes were in diplotene stage of meiotic prophase. In cryostat sections, the diaphorase reaction was negative.

In 15-day-old pubertal animals, the follicle formation was completed. The primary follicles showed a moderate positive NOS-reaction in the follicle cells. Later, in growing and antral follicles, both follicle and theca cells were stained. Oocytes remained negative at all developmental stages.

An increase in diaphorase staining was noted in the adult ovary. Besides in follicles, strong staining was demonstrated in luteal and interstitial cells (Figs. 4, 5, 6).

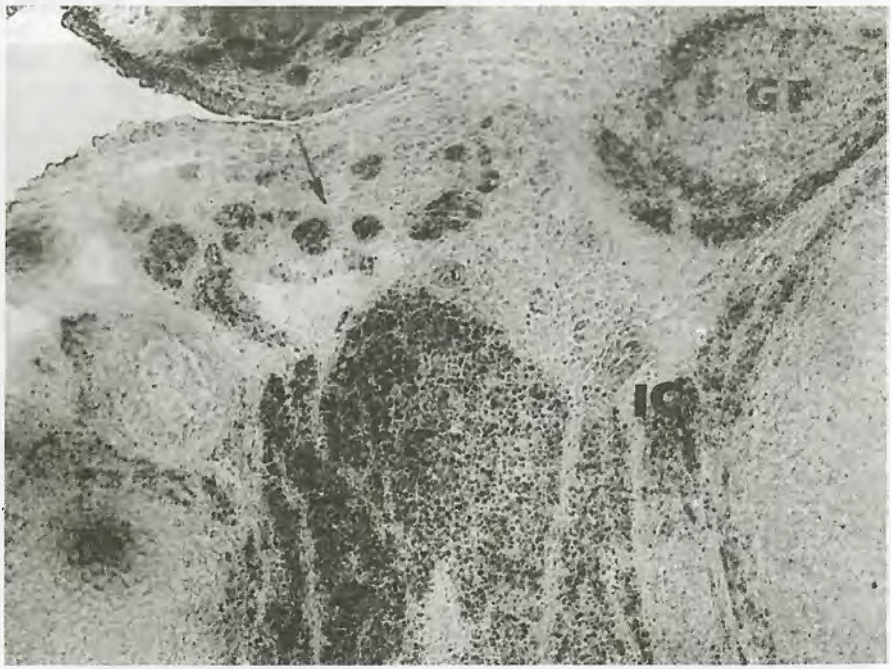

Fig. 4. Adult ovary NOS-reactivity in the follicle cells of primary follicles (→) and growing follicles (GF) as well as in the interstitial cells (IC) is present. ×160

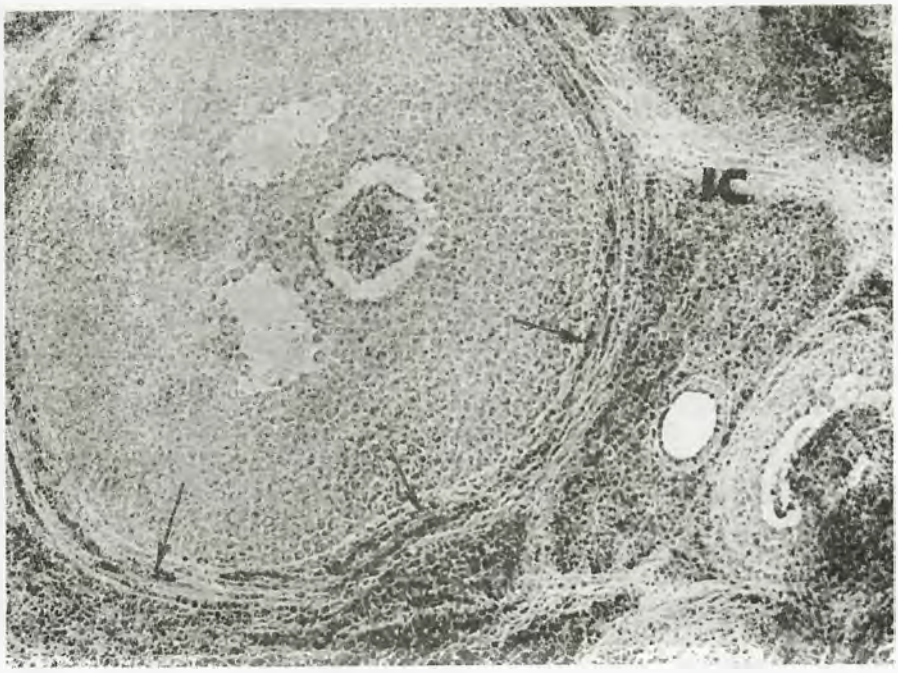

Fig. 5. Adult ovary theca cells (→) in an antral follicle and interstitial cells (IC) are diaphorase-reactive. ×160

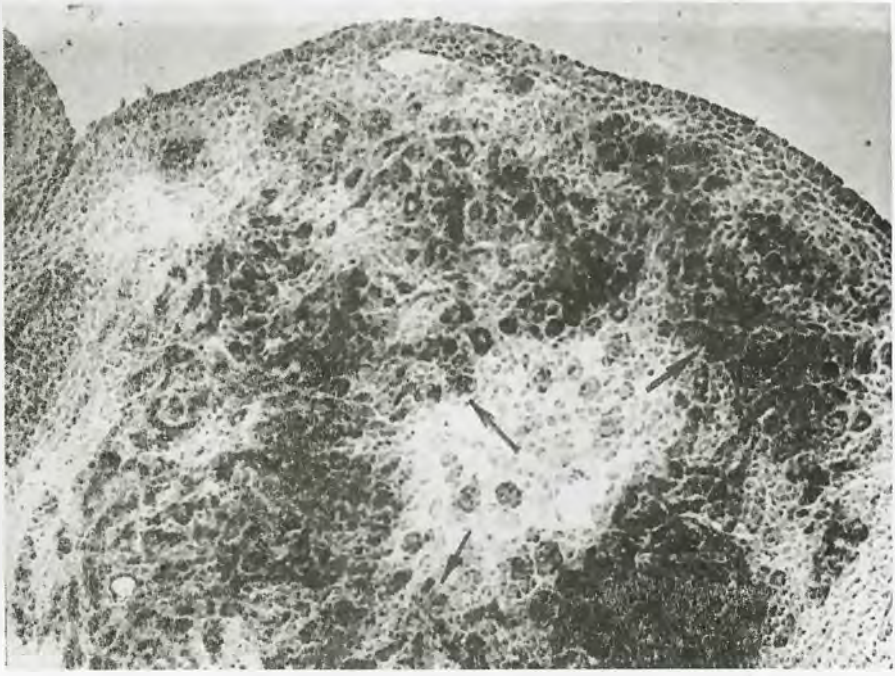

Fig. 6. A strong NOS-reaction is found in the luteal cells (→). ×160

Discussion

At present it is known that NOS is capable to exhibit a so-called NADPH-diaphorase activity which can easily be detected histochemically at the light microscope level.

In this study using NADPH-diaphorase activity of the enzyme, we were able to demonstrate the histochemical localization of NOS in developing rat gonads. It is interesting to note that the cellular diaphorase staining is correlated to the appearance and the differentiation of steroidogenic cells both in ovary and testis.

A diaphorase staining was present in fetal-type LC in neonates as well as in the second generation LC in pubertal and adult animals. In the ovary, the NOS was localized in all sreroidogenic cells: follicle cells in primary folicles, theca cells in growing and antral follicles, luteal and interstitial cells. High NOS-activity was observed at stages of high steroid production: in neonatal period for LC and starting from puberty to reach a maximum in adulthood for both testicular and ovarian steroidogenic cells.

Our results are consistent with previously reported data about immunocytochemically detected NOS-activity in rodent LC (A n g e l o v a et al., 1995, in press).

Nitric oxide is membrane permeable and may, therefore, have effects in surrounding cells, as well as in the cells in which it is formed (H o p e et al., 1991). Being an intra- and intercellular messenger, NO is evidently involved in steroidogenic process. Recently it was shown that all three NO synthase isozymes display significant sequence homology to only one other mammalian enzyme, cytochrome P-450 reductase (see L o w e n s t e i n, S n y d e r, 1992). Perhaps NOS and the cytochrome P-450 enzyme were linked in evolution, which fits with NOS displaying P-450 properties (M c M i l l a n et al., 1992)

In conclusion our results revealed the localization of NOS in gonadal steroidogenic cells thus suggesting a possible role for NO as a regulator of steroid hormone production and secretion.

A c k n o w l e d g e m e n t s

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Substance P and neuron-specific enolase in mouse testis. An immunocytochemical study

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An investigation was undertaken to visualize the two markers of neuroendocrine cells: neuron-specific enolase (NSE) and substance P (SP) in the mouse testis during ontogenesis. Mouse testes from neonatal, prepubertal and adult animals were studied. For immunocytochemical visualization of substance P (SP) and neuron-specific enolase (NSE), a combination of avidin-biotin-peroxidase complex (ABC) and PAP-techniques was applied. The SP and the NSE were observed to be present in the mouse testes at all stages studied, e. g. in both foetal and adult generations of srteroidogenic cells. The majority of Leydig cells showed a moderate to intense cytoplasmic immunostaining. The findings presented here confirm and extend the investigations concerning the nature of the human and some rodents Leydig cells. Together with other literature data our results suggest that the neuropeptides under study are demonstrated in the Leydig cells of almost all Mammalian species.

Key words: substance P (SP), neuron-specific enolase (NSE), Leydig cells, testis, mouse.

In the last few years numerous studies report that neuroactive substances such as renin [6], β-endorphin [14], methionin — enkephalin [11] and oxytocin [7, 8] were detected in gonads. The presence of a neurotransmitter, namely, substance P (SP) and of neuron specific enolase (NSE), a marker of neuroendocrine cells was demonstrated immunocytochemically in Leydig cells from different mammalian testes (human, hamster, mouse, guinea-pig) [1, 2, 3, 5, 9, 10, 11, 12]. The substance P has not been established in the rat testis, providing evidence for the existence of species — specific differences [2].

In mice, data about distribution of SP and NSE in the Leydig cells during ontogenesis are not available.

The aim of our study was to elucidate the localization of SP and NSE in the mouse testes during ontogenesis.

Materials and methods

Mouse testicular tissue was obtained from neonatal, prepubertal and adult animals, kept under standard conditions. The animals were sacrificed by decapitation under ether narcosis. Testes were rapidly removed in Bouin's solution for 24 to 48 h at room temperature (20 °C). After fixation, tissues were dehydrated and embedded in paraffin. Histological sections 6 to 9 μm thick were mounted on chromalum/gelatine precoated slides. For immunohistochemical visualization of SP and NSE, a combination of the peroxidase antiperoxidase (PAP) [13] and the avidin-biotin-peroxidase complex (ABC) — method according to D a v i d o f f and S c h u l z e (1990) was applied [4].

The SP-antiserum was obtained from INCSTAR (USA) and diluted 1:800. The NSE-antiserum was supplied by Dakopatts (Denmark) and diluted 1:200. A biotinylated anti-rabbit IgG, 1:250 (Vector, USA) was applied in the second step of the immunostaining. Then, a PAP complex (1:200, Dakopatts, Denmark) and an ABC-complex (1:250, Vector, USA) were used. Development of the peroxidase activity was performed with a solution containing 20 mg/100 ml 3,3'-diaminobenzidine-4 HCl (Sigma, USA) and H_2O_2 at a final concentration of 0,01 %. Controls: Sections were incubated with the primary antisera at their working dilutions, previously preabsorbed for 24 h at 4 °C with the corresponding antigens synthetic SP (Sigma) and human NSE (Polysciences) at a concentration of 20 or 100 μg/ml. No SP or NSE-like immunoreactivity was observed in the control sections.

Some histological sections were stained with haematoxylin eosin to compare the immunocytochemical findings with the histological structure of the gonads at the same stages of development.

Results and discussion

In the neonatal mouse testis the first foetal generation of Leydig cells was observed in the interstitium. At birth in the intertubular tissues the Leydig cells usually occur in groups or nests (Fig. 1). At this stage SP-like immunoreactive cells, single or in groups were situated between the primary tubules (Fig. 3).

The second proliferation of Leydig cells appeared from the 10th day after birth. Fully differentiated Leydig cells were found on the 15th day post partum. In the interstitium SP-immunoreactive cells, showing location and morphology of Leydig cells were identified (Fig. 4).

On day 30th after birth as in adult animals Leydig cells were present as cords or groups of large cells, situated between the seminiferous tubules (Fig. 2). A strong SP-like immunoreactivity in their cytoplasm was observed. Early spermatids exhibited an unspecific staining.

In all stages studied, the Leydig cells of both foetal and adult generations demonstrated a well expressed immunoreactivity for SP as well for neuron-specific enolase. The reaction product was localized in the cytoplasm of the steroidogenic cells, single, or in groups (Figs. 5, 6, 7).

Substance P and neuron-specific enolase negative staining in the testis were found during the first postnatal week, when a regression of Leydig cells was reported by some authors.

The results presented here confirm and extend previous investigations concerning man and some rodents (S c h u l z e, D a v i d o f f, 1987, 1991; A n g e l o v a et al.,

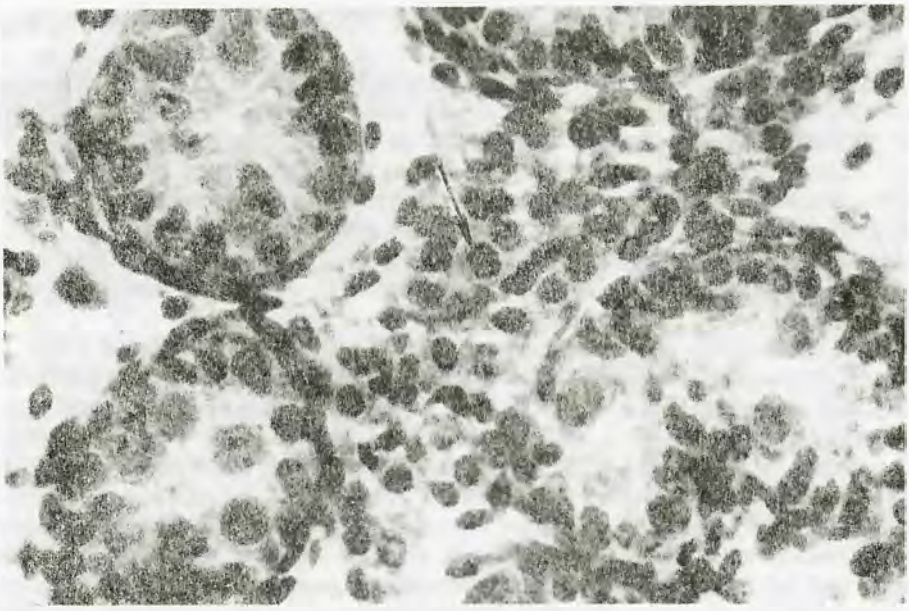

Fig. 1. Histological section: testis of a new-born mouse. Well differentiated single Leydig cells are present beween the sex cords (→). 160 ×

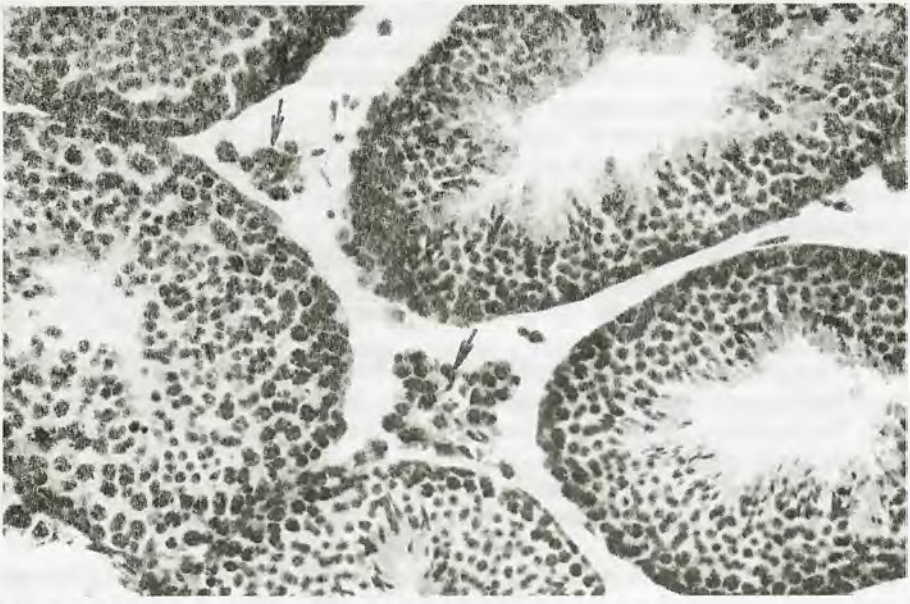

Fig. 2. Histological section: group of Leydig cells in the testis of an adult animal (→). 160 ×

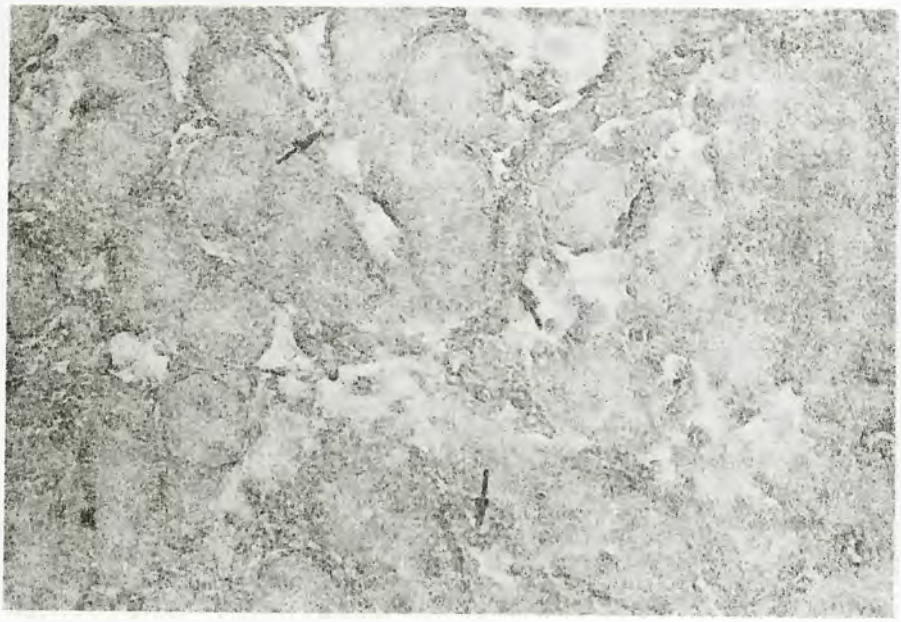

Fig. 3. SP-immunoreactive cells (→) in the testicular tissue of a new-born animal. 160 ×

Fig. 4. SP-immunostaining of the second generation of Leydig cells (10th day post partum) (→). 160 ×

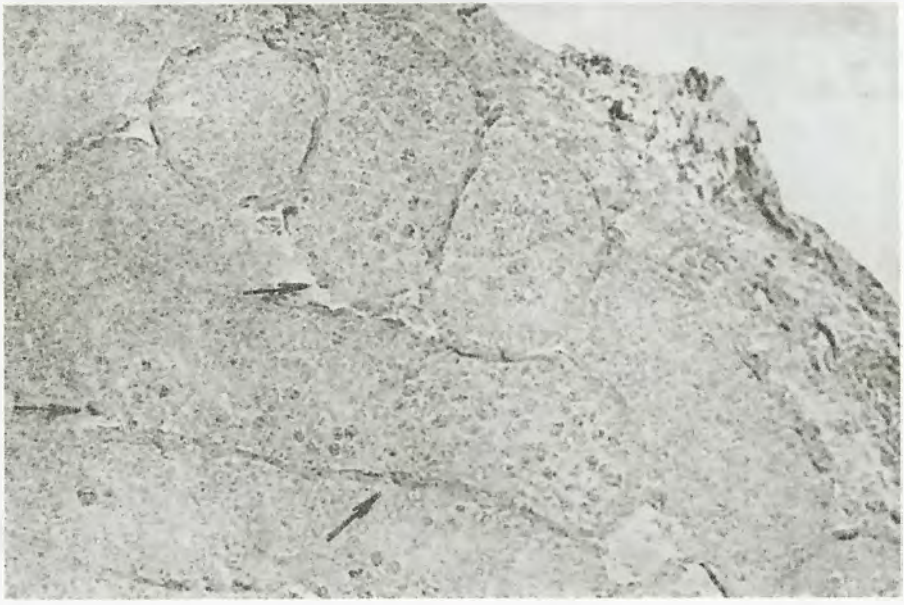

Fig. 5. NSE-like immunoreactive cells (→) of the second generation of Leydig cells (10th day post partum). 160 ×

Fig. 6. Testis from a 15-day-old animal. NSE-immunoreactivity in the Leydig cells (→). 160 ×

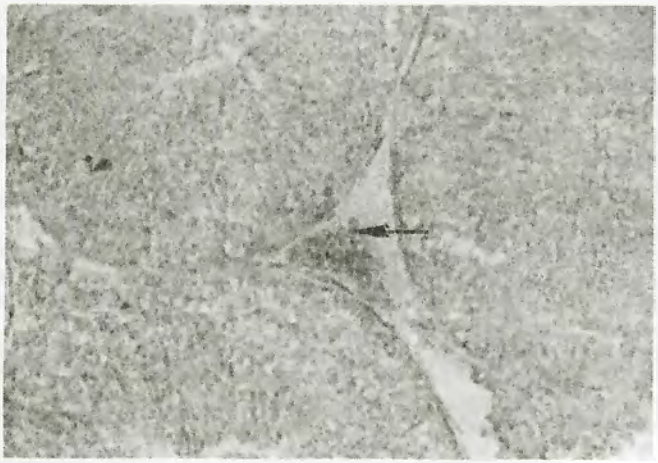

Fig. 7. NSE-immunoreactive Leydig cells (→) in the testis of an adult animal. 160 ×

1991). Our data suggest that the neuropeptides under study are demonstrated in the Leydig cells of almost all Mammalian species.

Our findings show a well expressed SP- and NSE-immunoreactivity in mouse Leydig cells during testicular development thus, completing their characteristics as neuroendocrine cells. Our data provide additional evidence for the neuroendocrine nature of Mammalian Leydig cells and confirm the data concerning their possible para- and autocrine function [3, 5, 9].

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Morphometric analysis of in vitro development of porcine immature granulosa cells stimulated by granulosa cell conditioned medium

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This paper examined whether ultrastructural changes characteristic of normal maturation or atresia of granulosa cells (GCs) accompany the steroidogenic alterations. Granulosa cells isolated from small porcine follicles (SGCs) unincubated or SGCs cultured in media supplemented with either serum or large follicle granulosa cell conditioned media (LGCCM) were studied. Under these conditions unincubated cells exhibited increase number of lysosomes, few osmiophilic lipid droplets and resembled atretic cells. In comparison serum cultured SGCs and specially LGCCM treated cells had increased osmiophilic lipid, mitochondria with vesicular cristae and smooth endoplasmic reticulum (SER) volume density. Progesterone secretion was stimulated in LGCCM treated cultures as compared to serum supplemented cultures. This suggests that LGCCM contains a factor(s) stimulating maturation while inhibiting ultrastructural correlates of follicular atresia.

Key words: ovary, granulosa, GC conditioned media, morphometry, progesterone.

It has been well demonstrated that porcine granulosa cell conditioned media (GCCM) possessed the capacity to produce and release a substance stimulating granulosa maturation (Š e b ö k o v a, K o l e n a, 1987). Conditioned media of granulosa cells obtained from small follicle (SGCCM) is at least equivalent to serum in supporting the steroidogenic activity. The effect of spent conditioned media obtained after 4-day culture of granulosa cells collected from small or large porcine follicles noted that the addition of GCCM increased progesterone (P) release in both systems studied. Higher amount of the active substance which stimulate progesterone secretion was released into conditioned media by granulosa cells from large follicles compared to small ones (Š e b ö k o v a, K o l e n a, 1987).

In the present study the effects of conditioned media, obtained from granulosa cells isolated from large follicles on the morphology of granulosa cells from small follicles were examined. Conditioned media of large follicle granulosa cells was used rather than spent media from small follicles because it contains greater stimulatory activity.

Materials and methods

Dulbecco's modified Eagle medium (DMEM) and tissue culture dishes were purchased from Flows Labs, UK; fetal calf serum (FCS) from Difco Labs, Detroit, Mich., USA. Human insulin (Pharmachim, Sofia) and human transferin (Sigma, St. Louis, Miss. USA) were gifts from Dr G. Miltchev. Other chemicals were of the highest purity commercially available.

Culture Procedures

Ovaries were obtained from pigs, 4-month-old or older, less than 20 min after slaughter and were immediately place on ice in a buffered salt solution containing 100 J. U./ml of penicillin, 100 µg/ml of streptomycin and 50 J. U./ml mycostatin. Granulosa cells from small follicles were isolated by the nonenzymatic needle puncture method described by Channing and Ledwitz Rigby (1975). Viable sells (as determined by trypan blue dye exclusion) were seeded in tissue culture dishes at a density 1.10^6 for 1 ml and cultured at 37 °C under a water-saturated atmosphere of 95 % air and 5 % CO_2 in DMEM supplemented with 50 J. U./ml penicillin, 50 µg/ml streptomycin, 2,5 µg/ml fungizone and 5 % FCS for 48 h. For the next 48 h granulosa cells were cultured in medium containing DMEM, supplemented with 25 % LGCCM, human insulin (J at 300mU/ml and human transferrin at 50 µg/ml.

Electron microscopy

At the end or culture period attached cells were scraped from the dishes with a rubber spatula and centrifuged at 600 xg. Unincubated freshly collected GCs and the pelleted cultured granulosa cells were embedded in gelly drop (30 % BSA + 50 % GA) and proceeded for electron microscopy.

Morphometric analysis

Morphometric analysis was performed on micrographs using the techniques of W e i b e l (1969). Fifteen micrographs were analyzed for each treatment group. Each cell constituent examined was outlined and the total area occupied by the specific organelle was recorded. Morphometric measurements were made, which involved stereological calculation of the volume density (the volume part occupied by a given compartment).

Progesterone assay

At the end of the culture the media were centrifuged and supernatants were stored at -20 °C until progesterone was assayed. The concentration of progesterone in the media from cultured granulosa cells was determined by the method of K a n c h e v et al. (1976) using rabbit antiserum (RD/4.10) at a dilution of 1:10 000.

Statistical analysis

Statistical difference was determined by Student's t-test.

Results

Studies from our laboratory have shown that freshly collected granulosa cells (control) had a large oval shaped nucleus surrounded by a small amount of cytoplasm.

After 48 h incubation in 5 % fetal calf serum the granulosa cells had flattened, appeared healthy and were fibroblast-like, whereas those cultured in 25 % LGCCM were epithelioid (Fig. 1, *a* and *b*). After 4 days incubation nuclear: cytoplasmic ratio was significantly greater in the freshly collected cells than in any other group (5 %

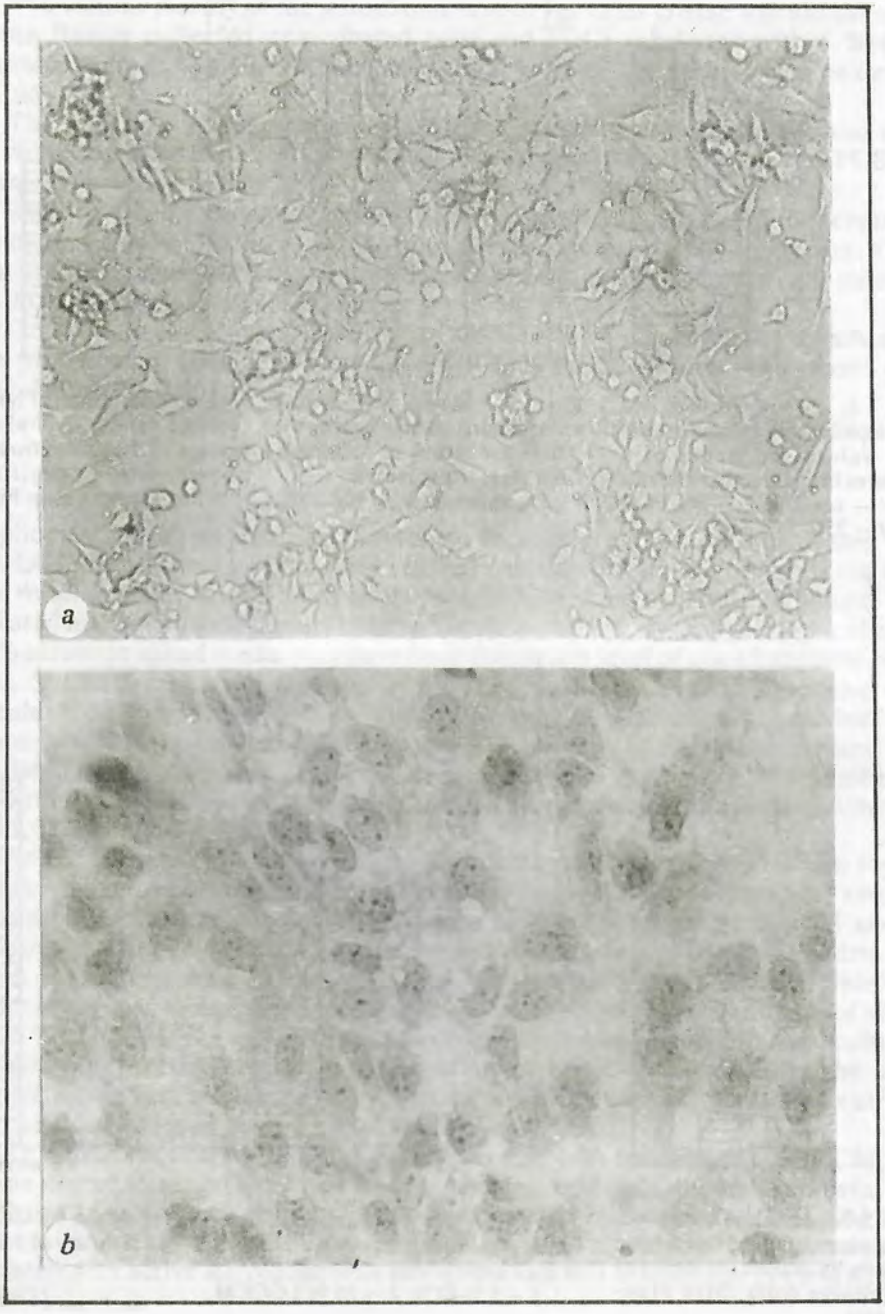

Fig. 1. Granulosa cells from small porcine follicles in 5 % FCS (fibroblast-like) (*a*) and 25 % LGCCM (epitheloid) (*b*)
a — 100×; *b* — 600×

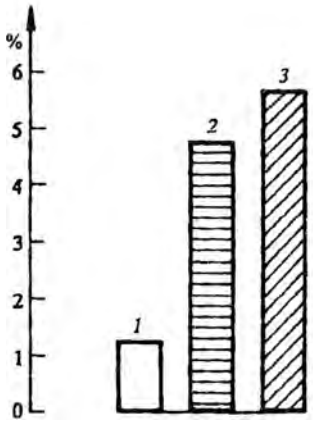

Fig. 2. Mean values of osmiophilic lipid volume density Vv. Values are means of 5 cultures from three experiments
1 — control; *2* — 5% FCS; *3* — 25% LGCCM

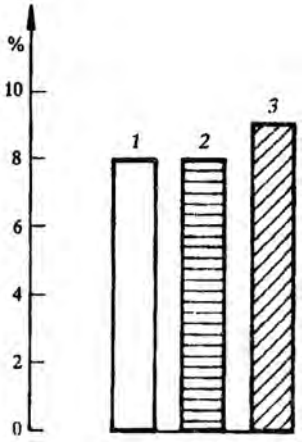

Fig. 3. Mean values of mitochondria volume density Vv. Values are means of 5 cultures from three experiments
Designations as in Fig. 2

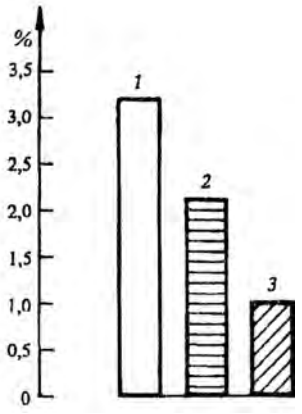

Fig. 4. Mean values of lysosome volume density Vv. Values are means of 5 cultures from three experiments
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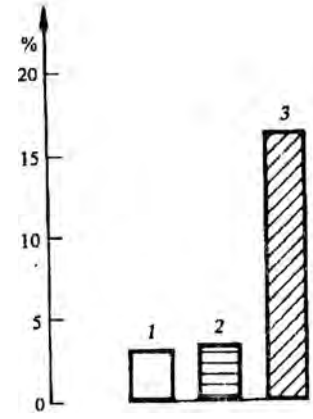

Fig. 5. Mean values of smooth endoplasmic reticulum volume density Vv. Values are means of 5 cultures from three experiments
Designations as in Fig. 2

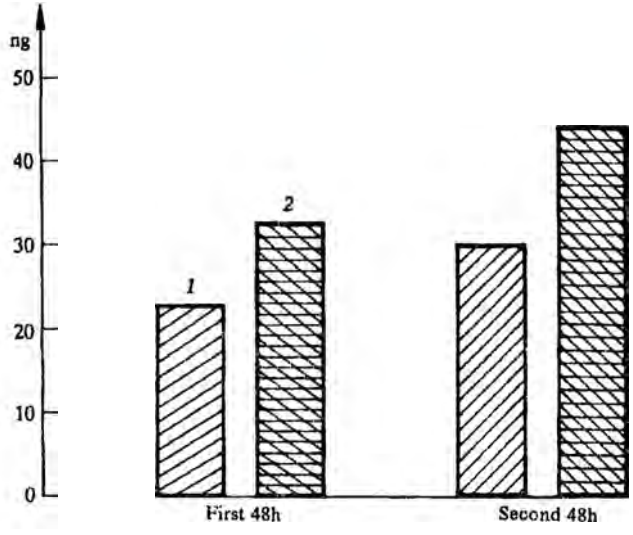

Fig. 6. Effect of FCS and LGCCM on P secretion by GCs from small follicles (ng/ml per 1.10^6 cells) Values are means of 8 cultures from 3 experiments
1 — 5 % FCS; *2* — 25 % LGCCM

FCS or 25 % LGCCM) and the number of microvilli increased in the case of 25 % LGCCM treatment (data not shown).

Lipid volume density was significantly higher after 4 days incubation in 25 % LGCCM in comparison with other treatment (5 % FCS) or in freshly collected unincubated granulosa cells ($p<0,001$) (Fig. 2).

The volume density of the mitochondria with vesicular cristae was almost equal in both freshly collected unincubated cells and SGCs incubated with 5 % FCS. Treatment with 25 % LGCCM increased slightly the mitochondrial volume density ($p<0,05$) (Fig. 3).

The lysosomal volume density was highest in freshly collected granulosa cells. The volume density decreased after incubation of granulosa cells with 5 % FCS and declined considerably after 25 % LGCCM treatment ($p<0,001$) (Fig. 4).

Smooth endoplasmic reticulum (SER) volume density appeared to increase in cells incubated with 5 % FCS, but the increase was not statistically significant. There was a dramatic increase in the volume density of SER in the granulosa cells cultured with 25 % LGCCM ($p<0,001$) (Fig. 5).

Granulosa cells incubated in 25 % LGCCM for 4 days secreted significantly more progesterone than cells cultured in 5 % FCS specially during the second 48 h of incubation ($p<0,001$) (Fig. 6).

Discussion

Morphometric analysis identified differences between granulosa cells incubated with 25 % LGCCM, serum or unincubated cells. Four-day-incubation of granulosa cells from small follicles in 5 % FCS or 25 % LGCCM alter their ultrastructure. The decreased nuclear volume density observed in the present study after 4 days incubation in 5 % serum or spend media may have been due to low level of gonadotropins in the media. Such a decrease was observed by Mc L e a n et al. (1986) after incubation with follicular fluid from large follicles. The appearance of microvilli on granulosa cell surface has been correlated with an increased number of LH receptors and LH stimulable adenylyl cyclase (J a r r i et al., 1994). An increase in the number of microvilli has been associated with granulosa cells maturation after incubation with 25 % LGCCM (W a d a, 1993).

One of the marked changes in granulosa cell morphology noted in this report was the change of osmiophilic lipid droplets, lysosomes, mitochondria, smooth endoplasmic reticulum content of granulosa cells cultured with 5 % FCS and in SGCs treated with 25 % LGCCM. The highest volume density of mitochondria was seen in culture treated with 25 % LGCCM. The most striking increase in lipid and smooth endoplasmic reticulum volume density was observed after treatment of SGCs with 25 % LGCCM. The lysosome volume density decreases when SGCs were cultured with serum and reaches minimal values after treatment with LGCCM. The large autophagocytic vacuoles seen in unincubated cells have been associated with atresia and resembled in vivo atretic cells (E l f o n t et al., 1992).

An important observation of our data was that cells incubated in LGCCM and in some degree in serum exhibited fewer signs of atresia than unincubated cells.

Progesterone secretion by SGCs was stimulated by serum and LGCCM to a greater extent on the 4th day than on the first 2 days and correspond to characteristics associated with active steroidogenesis and granulosa cell maturation (S t e w a r t et al., 1982).

In conclusion data presented in this paper demonstrated that spent media fron granulosa cells of large follicles alter small follicle granulosa cell morphology enhancing granulosa cell maturation as well as steroidogenesis.

Acknowledgements

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Mitogenic effect of prepubertal rat Sertoli cell secreted media on germ and somatic cells in vitro

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In recent years it has been established that Sertoli cells secrete a number of bioactive molecules which participate in the autocrine and paracrine regulation of the testicular function. Cultured Sertoli cells were isolated from postnatal rat aged 6 and 12 days. The Sertoli cell-conditioned media (SCCM-6 and SCCM-12) can markedly stimulate the proliferation of BALB/c 3T3 somatic cells and quiescent rat prespermatogonia.

Key words: Sertoli cells, cell culture, mitogenic factor (s), secreted proteins, germ cell proliferation.

It is known that Sertoli cells are involved in paracrine interactions with germinal cells in mammalian testis, and secrete a number of growth factors required for control of germ cell function (B e l l v e, Z h e n g, 1989; L a m b, 1993). Some of these growth factors as Seminiferous growth factors (SGF), Sertoli cell-secreted growth factor (SCSGF), Transforming growth factor (TGF-α and TGF-β), acidic and basic Fibroblast growth factors (aFGF, bFGF), have been isolated and characterized. Cell-cell interactions in mammalian testis are dependent on the requirements of different cell types at each stage of testucular development (M a r t i n o v a et al., 1993). Germ cells enter a resting period, which lasts from 17th day p. c. until 5th day p. p. when the mitosis reinitiates (H i l s h e r et al., 1974), and on 12th day p. p. germ cells enter meiosis.

Cell-cell interactions as well as Sertoli cell structure and functions during early prepubertal period are not well studied. The aim of present work was to investigate the effect of proteins secreted by Sertoli cell derived from 6th-and 12th-day-old rats on germ and somatic cell proliferation in vitro.

Materials and methods

Dulbecco's modified Eagle medium (DMEM), Ham's F-12, fetal bovine serum (FBS), tissue culture dishes and 96-well multidishes were supplied by Flow labs., U.K.; trypsin from bovine pancreas 3,1 U/mg and dialysis tubing of 8000 MW exclusion limit were

supplied by Serva; collagenase was obtained from Boehringer, Mannheim, F. R. G.; hyaluronidase from Fluka; [^{35}S]-methionine and [^{3}H]-thymidine were purchased from Amersham.

Sertoli cells were isolated from 6- and 12-day-old male Wistar rats according to a modification of the procedures of B e l l v e (1979). Decapsulated testis fragments were digested first with hyaluronidase (0,5 mg/ml), followed by a collagenase digestion (0,5 mg/ml). Both digestions were performed for 15 min each at 32°C in a shaking water bath and additionally pipetted for 5 min. The dispersed seminiferous cords were isolated by allowing them to sediment for 10 min and supernatant was discarded. The sediment was digested with trypsin (0,5 mg/ml) for 10 min under the same conditions and then was pipetted for 3 min. The dispersed cell were washed with 0,5 % BSA and filtered through a wire mesh grid (74 μm pore size). Sertoli cells were plated in tissue culture dishes at a density of (1÷1,5).1 000 000 cells/ml in DMEM/ Ham's F-12 (1:1) supplemented with 5 % FBS during the initial 24 h of cultivation. The cell cultures consisted of 85±5 % viable Sertoli cells. After 24 h of cultivation the cells were washed with DMEM and fresh medium without serum was added. The Sertoli cell-conditioned medium (SCCM) was collected on the 4th and 7th day after beginning of the cultivation. Purity of Sertoli cells reached about 90 % after 4 days of culture, while the germ cells were completely absent.

Samples of SCCM from 6- and 12-day-old rats (SCCM-6 and SCCM-12) in concentrations 8 ×, 4×, 2 ×, and 1× were tested for their ability to stimulate DNA synthesis in confluent quiescent culture of BALB/c 3T3 cells. The fibroblasts were grown to confluence in 96-well polystyrene multidishes (2.1000 cells per well) in 200 μl of DMEM containing 10 % FBS for 3 days. Subsequently, the fibroblasts were cultured 7 days in DMEM containing 0,5 % bovine serum. Different samples of SCCM without serum were tested for a further 24h in the presence of [^{3}H]-thymidine (37 kBq per well).

Two-dimensional gel electrophoresis was performed essentially as described by O'F a r r e l l (1975) with some modifications. The putative factor(s) was pre-characterized as a protein with a molecular weight over 8000, and sensitive to heat and trypsin treatment (M a r t i n o v a et al., 1988).

In each experimental group were used five male rats on 2,5 and 3,5 days p.p. The animals were decapitated and the testes were dissected free of tunica albuginea to obtain four pieces from each testis. The pieces were mounted on a highly permeable celloidin membrane having a plastic ring as a frame (J o r d a n o v, A n g e l o v a, 1984). The incubation medium consisted of DMEM (in controls) and different concentrations of SCCM-6 and SCCM-12, supplemented with 5 % FBS and [^{3}H]-thymidine in a dose of 74 kBq/ml. The specimens were incubated at 32 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 24 h.

To assess the beginning of DNA synthesis by [^{3}H]-thymidine incorporation in the quiescent prespermatogonia, the germ cells were scored for the presence of labels. The labelled germ cells were counted in every 3rd section of each explant. The final result for each group (n=5) was determined by the mean value of triplicate incubations.

The percentage of seminiferous cords containing labelled germ cells was evaluated in the same manner.

Results

During a week of cultivation Sertoli cells preserved their morphological characteristics, growth in monolayer and form distinct groups of cells. The nucleus is large with

irregular shape and invaginations. The heterochromatin accumulations are situated on the inner nuclear membrane, and several dense nucleoli are visible. The chromatin structure and nucleoli demonstrated functional activity of the cells.

Electrophoretic examinations of the proteins secreted in the spent media from Sertoli cells are shown on Fig. 1. 20—25 different proteins with m. w. between 10 and 94 kDa and 5 and 9 pI are synthesized. There is no differences between secretory activity of Sertoli cells isolated from 6- and 12-day-old rats.

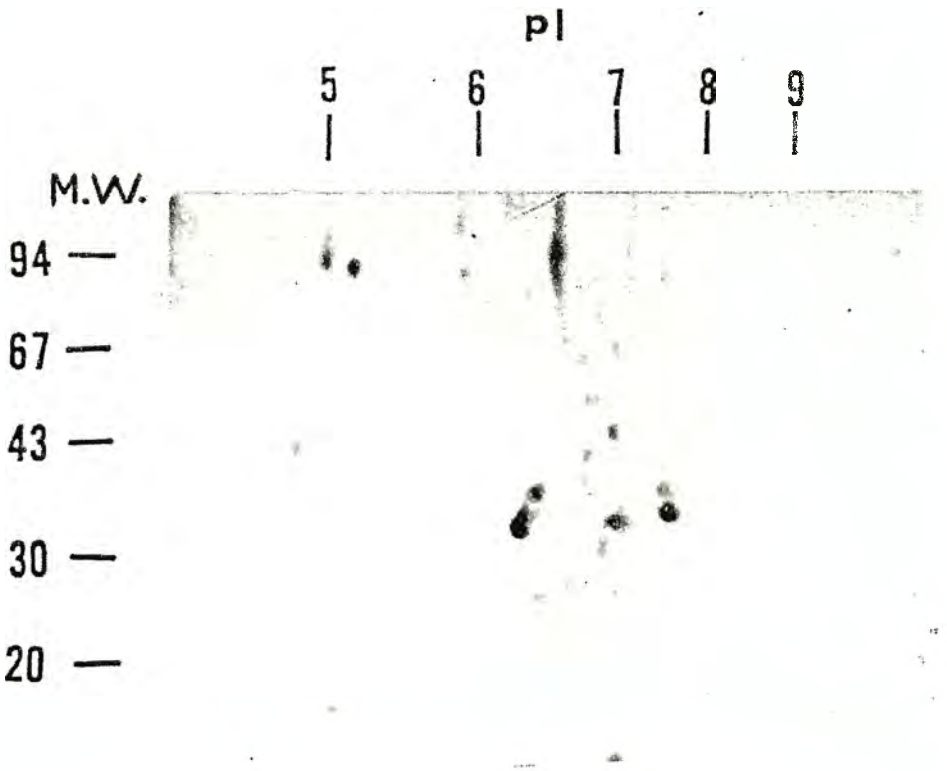

Fig. 1. Two-dimensional analysis of Sertoli cell secreted proteins.Cells were incubated in DMEM with [^{35}S]-methionine for 24h

The effect of different concentrations of SCCM-6 and SCCM-12 on the mitogenic activity of the BALB/c 3T3 cell line is shown in Fig. 2. SCCM-6 did not significantly increase cell proliferation of 3T3 cells in all tested concentrations (1×, 2×, 4×, respectively 10, 20, 40 μg/ml protein). A dose-response effect was observed in testing the same concentrations of SCCM-12. Maximum mitogenic effect was noted in 2× SCCM-12, when the [^{3}H]-thymidine incorporation was 7-fold over controls ($p<0,01$). The stimulation in the other concentrations was considerably high as well ($p<0,001$). In the group with heat treatment of 4 ×SCCM-12 a nearly 2-fold increase was registered in comparison with the control, but the differences were not significant ($p<0,25$). The mitogenic activity of SCCM-6 and SCCM-12 was trypsin sensitive (data not shown).

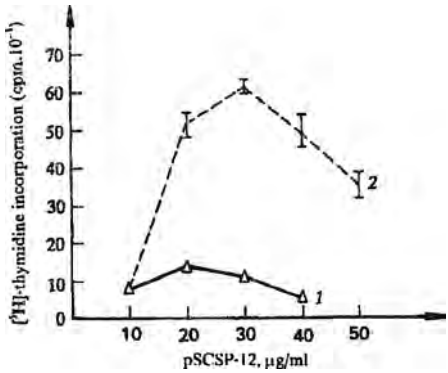

Fig. 2. Comparison between the effect of SCCM-6 (1×, 2×, 4×) and SCCM-12 (1×, 2×, 4×, 8×) on [³H]-thymidine incorporation by 3T3' fibroblasts in vitro
1 – 6 day; *2* – 12 day

Using the methods of seminiferous cord cultivation on the celloidin membrane we observed normal viability of prespermatogonia. From Fig. 3 it is evident that [³H]-thymidine is incorporated into the Sertoli cells as well as into prespermatogonia after incubation in SCCM-6 of testicular cords from a 3,5-day-old rat.

The effect of SCCM-6 and SCCM-12 on the mitotic activity of the germ cells from 2,5-day-old rats is expressed as percentage of seminiferous cords, containing labelled germ cells (Fig. 3) and as percentage of labelled germ cells (Fig. 4). SCCM-12 has no effect on the germ cells but SCCM-6 in a dose-dependent manner increases the investigated parameters up to 3-4-fold, compared to controls ($p<0,001$).

The mitogenic effect of SCCM-6 and SCCM-12 in the same concentrations is more expressed on the germ cells from 3,5-day-old rats (Fig. 5). Tested concentrations of SCCM-6 icrease significantly ($p<0,001$) up to 5-10-fold the percentage of seminiferous cords, containing labelled germ cells (Fig. 5) and the percentage of labelled germ cells (Fig. 6).

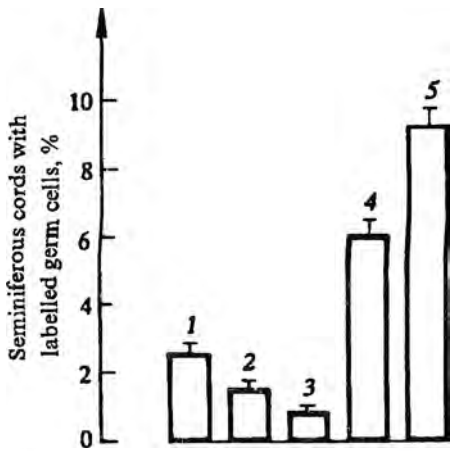

Fig. 3. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Seminiferous cords with labelled prespermatogonial cells
1 – DMEM (control); *2* – 4× SCCM-12; *3* – 2× SCCM-12; *4* – *4*× SCCM-6; *5* – 2×SCCM-6

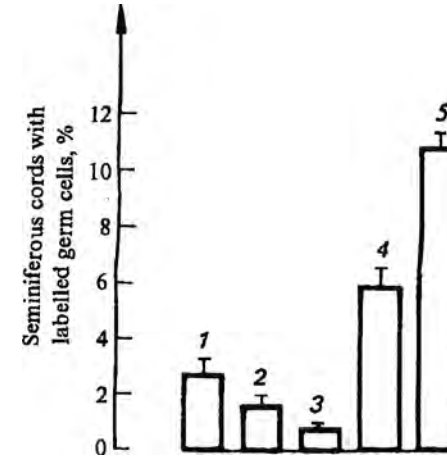

Fig. 4. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Percentage of labelled prespermatogonial cells
Designations as in Fig. 3

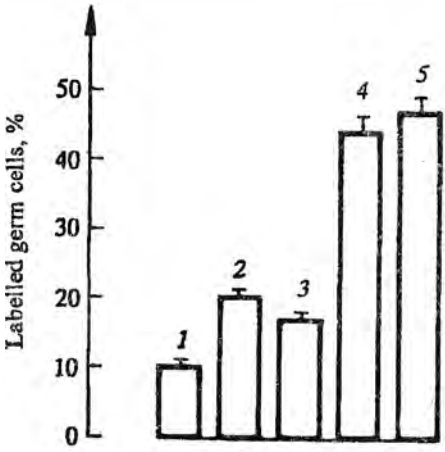

Fig. 5. Mitogenic effect of SCMM-6 and SCCM-12 on 3,5-day-old rat prespermatogonial cells. Seminiferous cords with labelled prespermatogonial cells
Designations as in Fig. 3

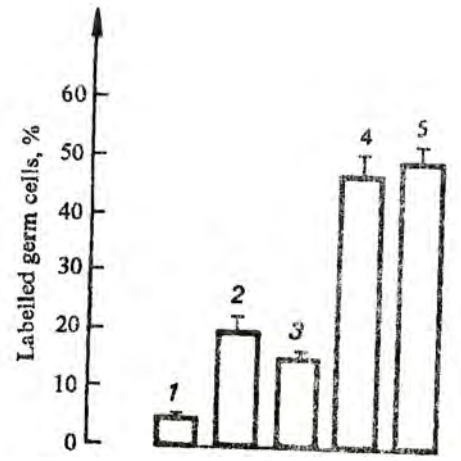

Fig. 6. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Percentage of labelled prespermatogonial cells
Designations as in Fig. 3

Discussion

In current study we have shown that Sertoli cell isolated from 6th- and 12th-day-old rat testis preserved their morphological and functional characteristics in vitro and secrete a number of products. Based on [^{35}S]-methionin labelling it is obvious that the Sertoli cell secretory products are of protein nature. Probably well preserved morphology and function of Sertoli cell in vitro is due to extracellular matrix secreted by Sertoli cell (M a t h e r, P h y l i p s, 1984) which creates the base for the cell attachments. In addition we have established that SCCM-12 markedly stimulate proliferation of somatic BALB/c 3T3 cells. 7-fold increase of [^{3}H]-thimidine incorporation over controls was shown after application of 20 μg/ml protein. In comparison SCCM-6 stimulate markedly prespermatogonial proliferation up to 10-folds over controls.

Based on the above mentioned results we could speculate that in spent media Sertoli cells secrete a mitogenic factor(s) which effectively stimulate proliferation of quiescent prespermatogonia. Mitogenic activity of SCCM from 6th-day-old rats is more effective on prespermatogonial proliferations since at this day prespermatogonia in the testis begin to divide, and they need a stimulus for the mitosis.

At the 12th day p.p. germ cells enter meiosis and the mitogenic activity of SCCM proteins is decreased. The putative mitogenic factor(s) appears to be proteinaceous with mol.weight over 8000 Da and sensitive to heat and trypsin treatment (K a n - c h e v a et al., 1990).

Having in mind the effect of purified SGF on prespermatogonial cell proliferation (M a r t i n o v a et al., 1992) it could be one of the most probable growth factor secreted by Sertoli cells. The coctail of growth factors secreted by Sertoli cell consist of SCSGF, FGF, TGF, IL-1, and the balance between different growth factors depends on the stage of differentiation of germ cells as well as of the rest cell types in the testis. Our results are in agreement with the hypotesis that Seroli cells mediate the

action of pituitary hormones and secrete a number of growth factors which participate in control of germ cell proliferation.

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Leydig cells — further immunocytochemical evidence for their neuroendocrine nature

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In mammals somatic cells secrete peptides regulating spermatogenesis and cell-cell interactions. In man and rodent testes a number of marker substances for neuroendocrine cells were demonstrated. Substance P, Neuron specific enolase, Mono-amine synthesizing enzymes (Thyrosine hydroxylase, *L*-Amino acid decarboxylase), Chromogranin A+B etc. Using immunocytochemical methods we were also able to demonstrate immunoreactivity for several other substances: Neurofilament protein 160, Neurofilament protein 68, Microtubule-associated protein (MAP-2), Calmodulin, Glutamate, Aspartate, Nitric oxyde synthase. Positive reactions for these antigens were found in the cytoplasm of the Leydig cells (LC) in three rodent species: hamster, mouse, guinea-pig. The uniformity in the localization of the investigated substances suggests that the revealed neuroendocrine markers and neuroactive peptides seem to be a basic equipment of mammalian LC. These findings provide further evidence for neuroendocrine nature of LC.

Key words: Leydig cells, neuroendocrine markers, immunocytochemistry, rodents.

Introduction

The gonadotrophins LH and FSH are the main endocrine modulators of the normal physiological functions of the testis, i. e. gametogenesis and steroidogenesis. In addition, steroids and peptides syntesized in the testis have been supposed to can mediate or modulate the actions of gonadotrophins [16]. By immunocytochemistry, a number of locally produced substances indentical or related to neurotransmitters or neurohormones, have been identified in the Leydig cells of the human and rodent testes: POMC-derived peptides [5, 12], the tachykinin substance P and neuron specific enolase [16,17, 1, 2], monoamine synthesizing enzymes thyrosine hydroxylase, aromatic *L*-amino acid decarboxylase and dopamine-β-hydroxylase, chromogranin A+B [8, 15, 4], serotonin [21].

On the other hand, it was established that the Leydig cells contain different growth factors or binding sites for them and neuroactive substances which possibly modulate testosterone production [14, 19].

Taking into account the considerations mentioned above, the present investigation was undertaken with the aim to verify the occurrence of other neuronal and

neuroendocrine markers in the Leydig cells of the three rodent species —mouse, hamster and guinea-pig): neurofilament protein 160 (NFP 160), neurofilament protein 68 (NFP 68), calcium-binding protein calmodulin, microtubule associated protein (MAP-2), glutamate, aspartate, the receptor for nerve growth factor (NGF-R) and nitric oxide synthase (NOS). We will try to get some information about the significance of those substances as autocrine/paracrine factors involved in the regulation of testicular functions in mammals.

Material and methods

Testes of sexually mature laboratory animals: mouse, golden hamster and guinea-pig, were studied. Tissue blocks were fixed in Bouin's fluid for 24 h at room temperature and then they were embedded in paraffin. Histological sections were mounted on chrom-gelatin precoated slides. For the immunocytochemical demonstration of the corresponding antigens, the peroxidase-antiperoxidase (PAP) technique with the avidin-biotin-peroxidase complex method according to D a v i d o f f and S c h u l z e [7] was applied.When polyclonal antibodies were used, the sections were treated with 1,2 % H_2O_2 in absolute methanol to inhibit endogenous peroxidase activity and then with 2 % normal rabbit or swine serum (to block the non-specific binding sites).

The sections were incubated with the primary antibodies or monoclonal antibodies for 48 h at 4 °C in a humid chamber. The following antibodies were used: 1) Mouse monoclonal antibodies directed against NFP 160 (Sigma, 1:200), NFP 68 (Sigma, 1:200),Calmodulin (Sigma, 1:200), MAP-2 (Sigma, 1:200), NGF-R (Sigma, 1:100); 2) Rabbit polyclonal antisera: anti-aspartate (Sigma, 1:200); anti-glutamate (Sigma, 1:1000); anti-nitric oxide synthase-I (Dr Mayer, Graz, Austria, 1:1000). The second antibodies, biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG (Dakopatts) and ABC-complex (Vector 1:250), the mouse PAP-complex (Dakopatts 1:100) or rabbit PAP-complex (Dakopatts, 1:200) were applied.

Development of the peroxidase activity was performed with a solution containing 0,2 % 3,3'-diaminobenzidine-4 HCl (Sigma) and H_2O_2 at a final concentration of 0,01 %.

As controls, sections were used in which primary, secondary or tertiary antibodies were replaced by PBS.

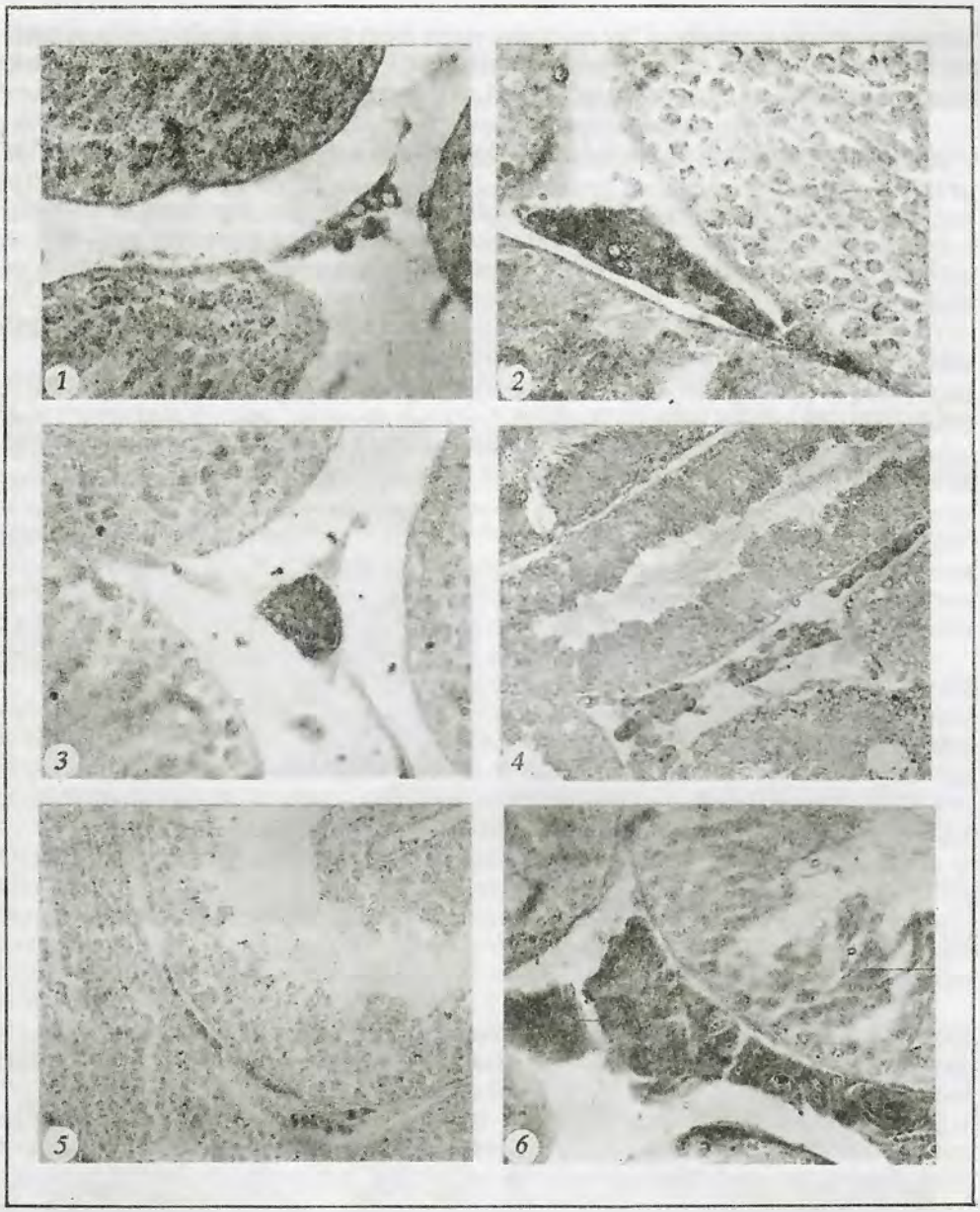
The staining intensity of every section was estimated at pluses (points) by two examiners independent of one another.

Results

Leydig cells of the rodent testes under study present a moderate to strong immunoreactivity for NFP 160, NFP 68, MAP-2, glutamate, aspartate, calmodulin, NGF-R and NOS.

In all cases immunoreactivity was localized within the cytoplasm of the Leydig cells. It is important to note that the cytoplasm of a great number of cells was stained while that of the remaining steroidogenic cells of the same cluster or in other groups is negative (Figs 4,9).

Secondly, differences in immunostaining intensity among testicular material from hamster, mouse and guinea-pig were observed. Most of the Leydig cells in mouse testis possess a stronger immunoreactivity for all tested antigens (Figs. 2,6,8). In



Figs. 1, 2. MAP-2 immunoreactivity in hamster (Fig. 1) and in mouse (Fig. 2) Leydig cells (LC). Differences in staining intensity among the LC in the same group are observed. ×400
Figs 3,4. Calmodulin immunoreactivity. In hamster testis (Fig. 3) a stronger reaction than in mouse LC (Fig. 4) is present. ×200
Figs 5,6. Neurofilament protein 160 expression in LC of guinea-pig (Fig. 5) and mouse (Fig. 6) testes. ×160, ×400 respectively

hamster testis the intensity of the reactions varies from strong to moderate. In guinea-pig there were large groups of positive cells, but in other clusters of steroidogenic cells only a small number of them showed posititve staining (Figs 5,10).

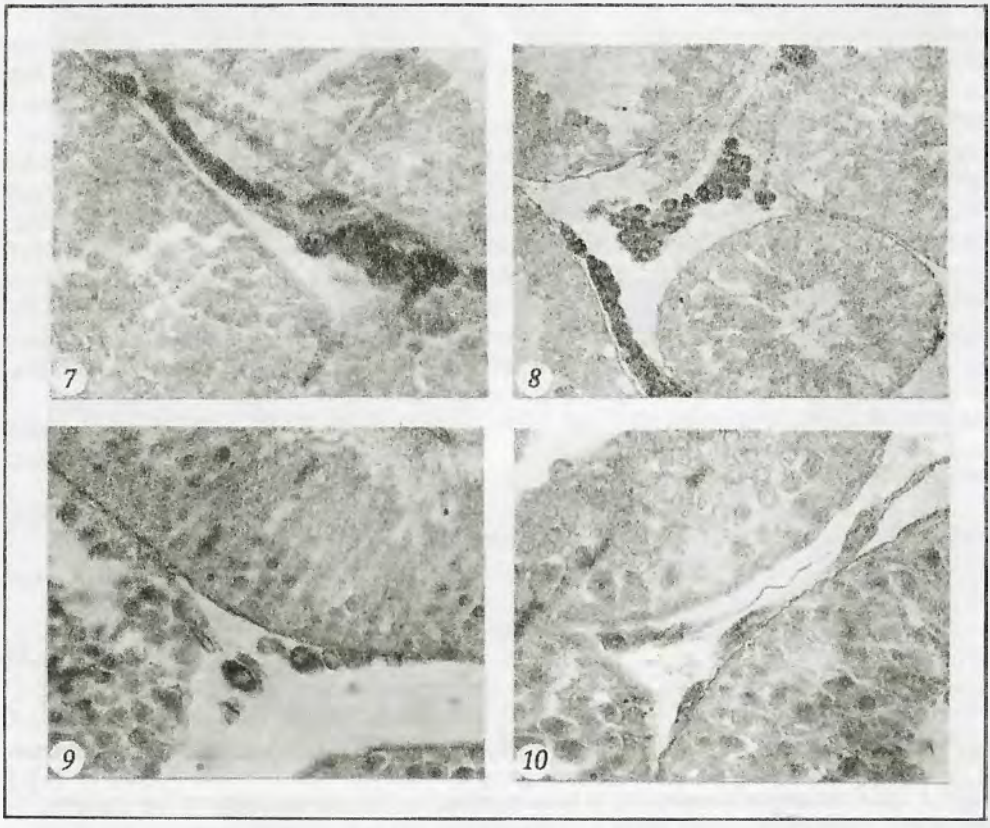
The MAP-2 reaction was stronger when the double amplification technique was applied and the staining product had predominantly a perinuclear distribution (Figs 1,2).

The calcium-binding protein calmodulin showed a similar localization in Leydig cell cytoplasm and a strong to moderate immunostaining was present (Figs 3,4). The positive immunoreactivity for NGF-R showed the presence in the Leydig cells of binding sites for a specific growth factor for the nervous system.

Concerning neurofilament protein 160 and neurofilament protein 68, a stronger reaction was observed in the case of the NFP 160 (Figs 5,6).

Both reactions for glutamate and aspartate were well expressed in mouse, hamster and guinea-pig Leydig cells (Figs 7,8).

In our material we were able to demonstrate immunocytochemically the occurrence of NOS in Leyding cells of the three species studied (Figs. 9,10).



Figs 7,8. Glutamate immunoreactivity in LC of hamster (Fig. 7) and mouse (Fig. 8) testes. In both cases a strong immunostaining is observed. ×400, ×200 respectively
Figs 9,10. NOS immunostaining in hamster (Fig. 9) and guinea-pig (Fig. 10) LC is demonstrated. ×400

Discussion

The present study show that Leydig cells in mouse, hamster and guinea-pig testes possess a great number of neuropeptides and neurotransmitters.

The immunocytochemical data about the existence of neurofilament proteins — NFP 160 and 68, MAP-2, glutamate and aspartate in Leydig cell cytoplasm point to common characteristics with nerve cells. Our findings enlarge the already published results concerning human testis [8]. The MAP-2, a protein typical for nerve cells is also demonstrated in our material as well as in rat Leydig cells [9]. It seems that the neuropeptides expressed in Mammalian Leydig cells are a common basic equipment of testicular steroidogenic cells probably stabilizing their structure and function [15].

In the three species investigated Leydig cells show a positive staining for calmodulin which is a typical component of neuroendocrine cells. It is postulated that Ca^{++}interacts with intracellular Ca^{++}-binding proteins such as parvalbumin, calbindin D-28 K, S-100 protein and calmodulin which then regulate the intracellular calcium concentration and translocation [11]. In the testis, calcium is thought to be involved in the production and secretion of testosterone and in the regulation of spermatogenesis [20].

The positive NGF-R immunoreactivity suggests that rodent Leydig cells possess receptors for this growth factor thus supporting the opinion about neuroendocrine nature of Leydig cells. Possibly, NGF is involved in autocrine regulation of testicular functions as well as other growth factors: EGF, TGF-β_1 [19, 10].

All proteins investigated by us co-exist in rodent Leydig cells, however in different concentration. Their different expression may be species-specific, but most probably it is related to the different functional state of the steroidogenic cells. The finding that in the same tissue section or in the same group, LC show a positive immunocytochemical reaction or remain unreactive, points to the second possibility.

Recently it was established that nitric oxide is a major messenger molecule regulating blood vessel dilatation and serving as a neurotransmitter [13]. NOS is activated by calcium which binds calmodulin as an enzyme cofactor [13]. By immunohistochemistry this enzyme was not identified in liver, heart, spleen, kidney, testis, thymus etc [6]. In our investigations the localization of NOS in the Leydig cell cytoplasm point to a possible action of nitric oxide as an intercellular messenger in steroidogenic process.

The current findings together with already established expression of a great number of bioactive substances by Mammalian Leydig cells provide further evidence for their neuroendocrine nature and raised the question about the functional significance of peptides mentioned above. A modulatory action of substance P, a tachykinin, on in vitro release of testosterone by hamster Leydig cells was earlier reported [3]. But the complex process of spermatogenesis is unlikely to be fully understood until all the factors involved in its control become known. Most probably, the neuropeptides, calcium-binding proteins, growth factors (or binding sites for them) identified immunocytochemically in the Leydig cells play a crucial role in testicular autocrine/paracrine mechanisms [5, 18].

Acknowledgements

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Effect of leucotriene E4 on the microvascular endothelium: an ultrastructural study

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Terminal vessels in the rat ciliary processes, the mucosa of the stomach and large intestine were studied by electron microscopy after exogenous (topical) application of Leucotriene E4.
All arteriolar vessels expressed morphological signs of contraction with bulging of the nuclear region of the endothelial cells (EC) into the lumen, deep basal evaginations of their extranuclear region and swellings of the vascular smooth muscle cells. In venules this phenomenon was accompanied by an increase in various-sized luminal evaginations of EC. The majority of capillary EC appear to be very rich in pinocytotic vesicles and Weibel-Palade bodies. Postcapillary and venous EC show an increased number of large-diameter vesicles. Endothelial gaps were encountered only in ciliary process venous-limb capillaries. Judging from the morphological alterations in EC, our data confirmed Leucotriene E4-mediated histamine-like effects on the endothelium of terminal vessels.

Key words: microvascular endothelium, luminal evagination, contraction.

Increased vascular permeability is one of the major effects of imflammatory mediators, which are a chemically nonhomogeneous group. Leucotrienes (LTs) are derivatives of arachidonic acid and LTE4 is a representative of the group of peptidoleucotrienes (LTC4, LTD4 and LTE4), that are known also as the slow-reacting substance of anaphylaxis (SRS-A). LTs are considered as mediators of antigen-induced inflammation [6]. Regardless of the nature of inflammatory agents, the sequence of microvascular events of the acute inflammation is characterized by arteriolar constriction, increased postcapillary vessel permeability and endothelial contraction [7, 8] leading to tissue oedema and migration of blood-borne cells [9]. Pharmacological studies have also shown that the effect of peptidoleucotrienes is similar to that of histamine, although LTs are more powerful [3].

The aim of the present study was to evaluate the in vivo effects of exogenously applied LTE4 on the ultrastructure of microvessel endothelium in different tissues of high metabolic activity such as the gut mucosa ansciliary processes.

Material and methods

Four adult Wistar rats were used. Animals were anaesthetized with sodium pentobarbital (Sagatal, 60 mg/kg i. p.). Leucotriene E4 (Merk Frost, Canada Inc,

kindly provided by Dr J. Rokach) in a single dose of 5 g/ml dissolved in saline was injected into ligated segments of the stomach and large intestine and left there for 4 minutes. Topical application of the same substance on the conjunctival surface of the left eye for a period of 4 minutes was applied too. After immediate in situ fixation of gut segments and the conjunctival surface for 5 minutes with 3 % glutaraldehyde, pieces of the stomach and large intestine, as well as of the ciliary body (1 mm thick and 3 mm long) were fixed in the same fixative for 2 hours at 4°C. After postfixation in 2 % osmium for 2 h at 4°C the specimen was processed for conventional transmission electron microscopy.Four control rats were used. A corresponding volume of saline was applied on controls. Tissue was taken also from the contralateral eye of the experimental animal. Observations were made on an Opton EM Turbo electron microscope.

Results and discussion

As a common effect of LTE4 we found well developed morphological signs of contraction in all segments of the microvascular system in the gut and ciliary processes after exposure to LTE4. Preterminal arterioles were heavily contracted. The lumen of these vessels was narrowed with the elastic membrane showing numerous deep curves.

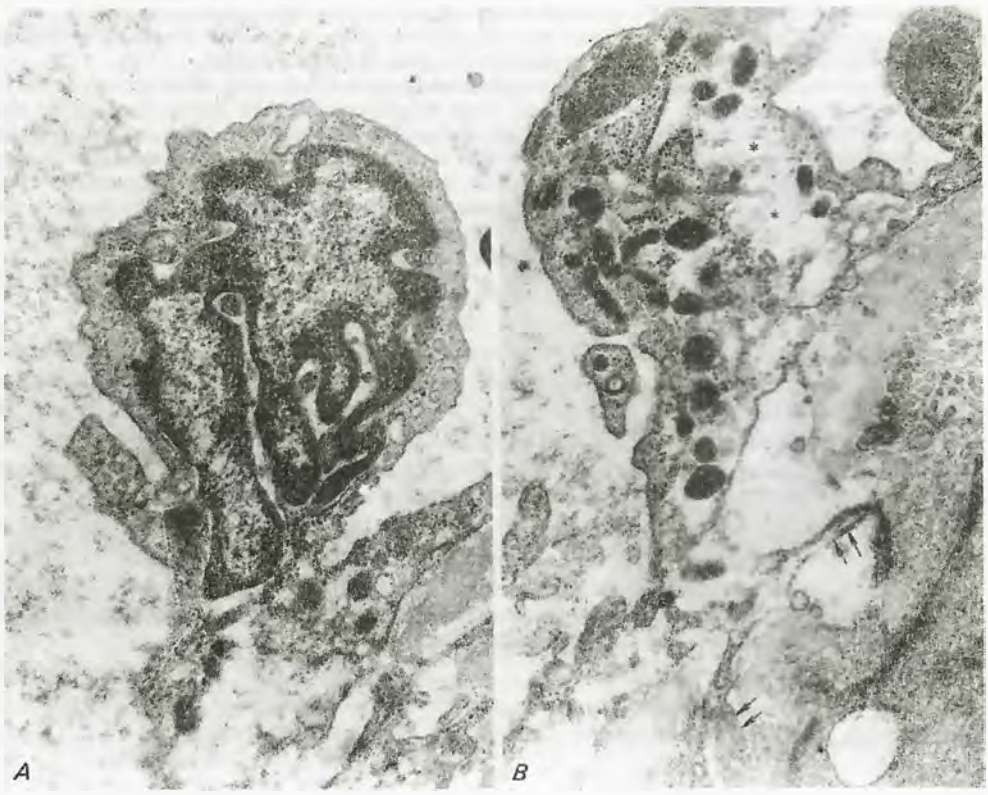

Fig. 1. An arteriole in the gastric mucosa. ×33 000
A – bulging of the nuclear region of the endothelial cell into the lumen; note the numerous invaginations of the nuclear membrane; *B* – accumulation of Weibel-Palade bodies in the peripheral zone of the cell, destructions of the endothelium are observed as lucent areas (asterisk), basal protrusions (arrows) penetrate through the elastic lamina

Endothelial cells had numerous deep evaginations into the lumen, especially at their nuclear region(Fig. 1. *A*). The nuclei exposed numerous deep invaginations too (Fig. 1, *A*) and the perinuclear space was considerably wide. Peripheral zones of EC and especially at the regions of interendothelial junctions were very thin although gaps were never observed. Basal protrusions of these zones penetrated into the deep folds of the ondulated elastic lamina (Fig. 1, *B*). Certain destructions were observed as optically empty spaces (Fig. 1, *B*). Weibel-Palade bodies were numerous (Fig. 1, *B*).

Terminal arterioles showed similar ultrastructural changes, as well as the appearance of vacuole-like spaces.

The lumen of capillaries was narrowed or almost occluded with bulgings of the nuclear zone into the lumen. Micropinocytotic vesiculation was high and mostly present in fenestrated vessels. Similar findings were true for postcapillary and venular EC. However the lumen of venous-limb vessels was filled with numerous evagintions of various size and length (Fig. 2). Endothelial gaps were seen only in ciliary process

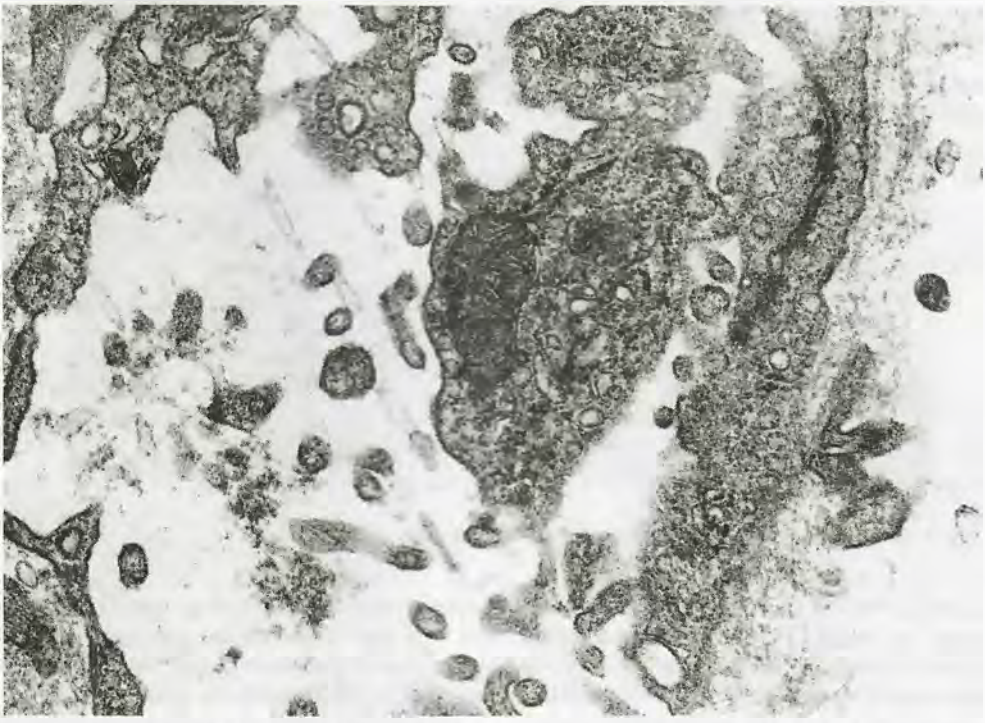

Fig. 2. Postcapillary venule. Lumenal evaginations of the endothelial surface. ×30 000

postcapillary vessels (Fig. 3).Numerous Weibel-Palade bodies were observed (data not shown), although normally they are very rare or absent in ciliary process endothelium.

Our results show that the effect of LTE4 on the microvascular EC is similar to that of histamine and serotonin [1, 2]. This suggests that EC as a whole react ultrastructurally uniformly to a variety of vasoactive inflammatory agents. These results are consistent with previous pharmacological studies which have shown that

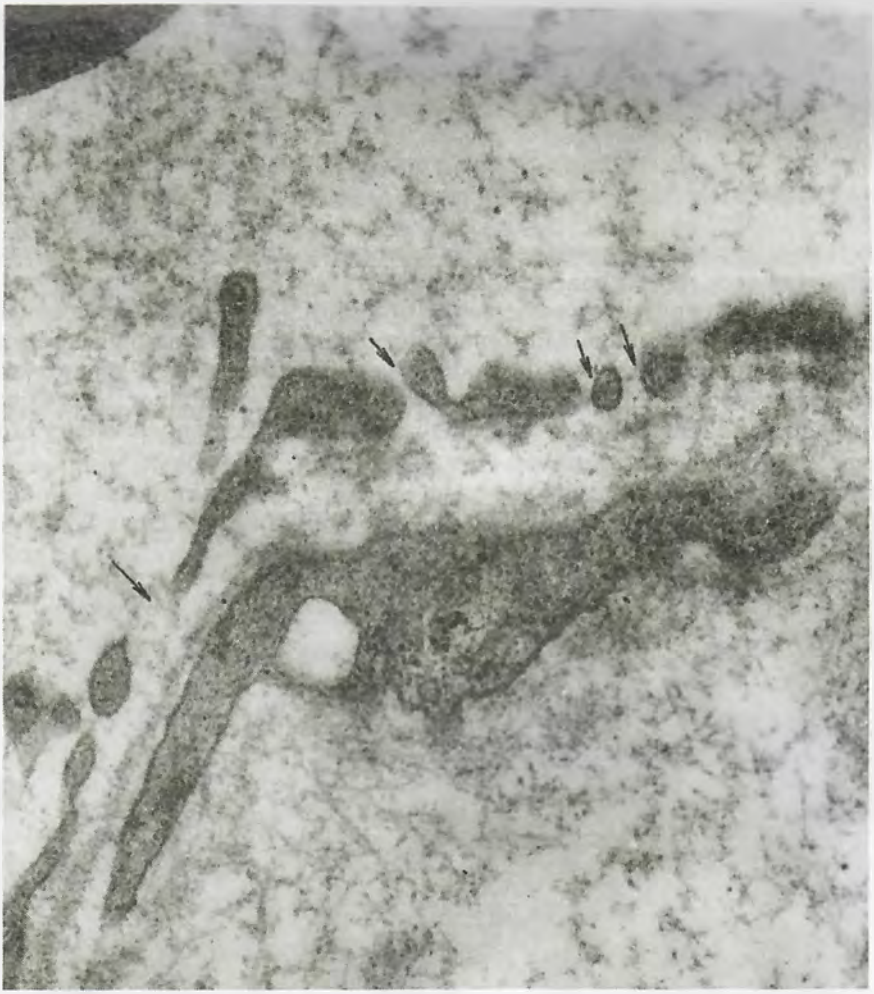

Fig. 3. Gaps (arrows) in ciliary process fenestrated capillary. ×50 000

along with the contraction there is an increase in capillary filtration coefficient after application of LTs [11]. Increased vesiculation may also lead to enhanced transvascular fluid exchange. Reports show that enhanced pulmonary vascular resistance and increased vascular permeability during anaphilactic and endotoxic shock are largely caused by LTs [5, 10], while application of LT-antagonists leads to a substantial pressure fall [4]. Postcapillary EC seem to respond differentially to the "disrupting" effect of LTE4 in different vascular beds as found in cilliary processes. Weibel-Palade body accumulation may increase pro-coagulant activity of vessels.

Our morphological observations after local application of LTE4 suggest that there may be a decrease in blood flow and increase in vascular resistanse due to vessel contraction. Vascular permeability may be enhanced due to increased vesiculation in endothelial cells.

Further studies are needed to assess the role of LTs on different components of the vessel wall.

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Stress ulcer : a morphological approach

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The effects of starvation and cold-restraint stress on the rat stomach mucosa were investigated. Mucosal damage was evaluated using light, transmission (TEM) and scanning (SEM) electronmicroscopy. Light microscopy revealed hemorrhagic areas as well as cellular damage in the mucosa. At TEM level, severe degenerative changes were especially observed at parietal cells and chief cells of the fundic mucosa. SEM demonstrated a totally necrotic appearance of the mucosa at the ulcer region with widened gastric pits devoid of lining epithelium. We conclude that experimental stress conditions produce severe gastric mucosal damage.

Key words: stress ulcer, gastric mucosa, morphology, microscopy.

Introduction

In literature, it is mentioned that stress conditions cause severe damage in the gastric mucosa [1, 2, 3] which was thought to occur as a result of autonomic nervous system activation [2, 3, 4]. In our study, we want to investigate rat fundic mucosa in an experimental stress ulcer model at light, transmission and scanning electronmicroscopy and we want to relate these morphological observations to the ulcer pathophysiology.

Materials and methods

Wistar albino rats of both sexes (200-250 g) were fed a standard diet and water ad libitum. The control group (n=8) was deprived of food for 3 h. Animals in the stress group (n=13) were immobilized in plastic restraining devices at 2-4 °C for 3 hours following a 48 h of starvation period. After stress application, animals were decapitated. Fundus material was taken from the macroscopically defined ulcer regions. For light microscopy, fundus specimens were fixed in 10 % formalin solution. The sections from paraffin blocks (4-5 μm) were stained with Haematoxylin-Eosin and investigated at Olympus BH-2 light microscope. The stomach was also examined macroscopically to determine the ulceration index as described by S e n a y et al. [5].

For transmission electronmicroscopic (TEM) examination, tissue specimens were fixed in 5 % phosphate-buffered glutaraldehyde and postfixed in 1 % OsO_4 solution for 1 hour. Following Epon 812 embedding, semithin sections of 1 μm were stained with Azur B and examined at Olympus BH-2 light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at a JEOL 1200 SX transmission electronmicroscope.

For scanning electronmicroscopy (SEM), tissue samples, fixed and dehydrated as mentioned above, were transferred into amyl acetate solution and then dried in a critical point dryer (Biorad). Specimens were coated with gold using a sputter-coater (Biorad 502 SC) and examined in a scanning electronmicroscope (JEOL 5200).

Results

Control group

Light microscopical examination of control group revealed a normal rat fundic mucosa. Intact surface mucous cells were columnar. Gastric pits were of the expected depth. Neck portion of the gastric glands consisted of mucous neck cells while their bases contained parietal cells, chief cells and enteroendocrine cells. Mucous neck cells were irregular in shape with a basally located nuclei. Parietal cells were pyramidal with one or two centrally located nuclei. Their cytoplasms were intensely eosinophilic. Chief cells were noticed with a rounded and intense basophilic cytoplasm (Fig. 1).

Fig. 1. Control group: normal state of rat fundic mucosa is observed. H. E. stain, ×132

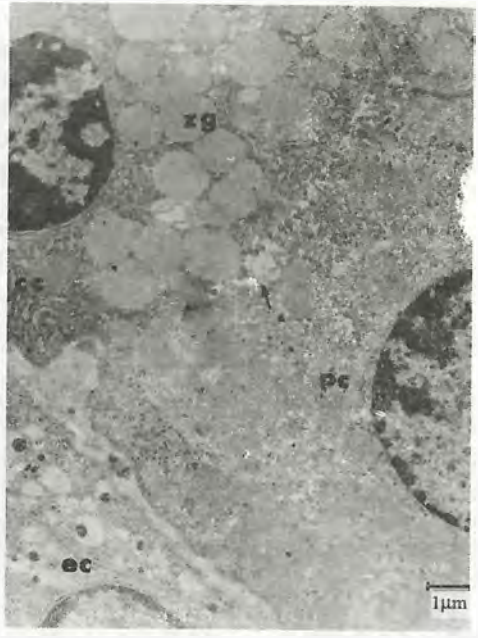

Fig. 2. Control group at TEM level: parietal cells (pc), chief cells (cc), endocrine cells (ec) reflect a normal ultrastructure.

Arrow – intracellular secretory canaliculi; zg – zymogenic granules

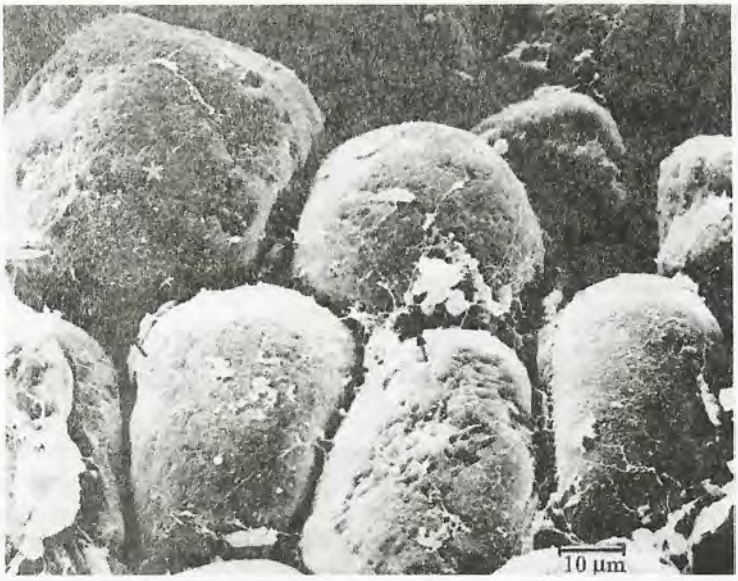

Fig. 3. SEM investigation of the control group: tightly bound polygonal surface mucous cells (*) and narrow openings to the gastric pits (arrow)

At TEM level, parietal cells were distinguished with their charactheristic intracellular secretory canaliculi lined by numerous microvilli. Adjacent to canaliculi, an extensive tubulovesicular system was observed. Numerous mitochondria were present around the intracellular secretory canaliculi. Chief cells represented typical protein-secreting cell ultrastructure with extensive granular endoplasmic reticulum and well-developed Golgi complex. Endocrine cells were within the fundic glands with their characteristic secretory granules. Granular endoplasmic reticulum and Golgi complex were sparse (Fig. 2).

Scanning electronmicroscopical investigation of the control group revealed closely packed, polygonal surface mucous cells and narrow opening to the gastric pits (Fig. 3)

Stress group

At light microscopy, stress group fundic mucosa showed a prominent cellular damage. Disrupted surface mucous cells were vacuolated and had pyknotic nuclei. In addition to luminal damage, cells lining the fundic glands were also disrupted and exfoliated (Fig. 4). In that group, distinct extravasated free erythrocytes both in lamina propria and luminal surface reflected an obvious hemorrhage which was also observed at muscularis mucosa, submucosa and muscularis layers indicating a true ulcer formation (Figs. 4, 5) which was confirmed by an ulcer index of 13,5—3,3 mm.

Ultrastructurally, at TEM level, we especially investigated parietal and chief cells. Pyknotic nuclei of parietal cells presented prominently enlarged perinuclear spaces (Fig. 6). In contrast to extremely widened intracellular secretory canaliculi reflecting an extreme acid secretion, tubulovesicular system was restricted (Figs 6, 7, 8). Numerous mitochondria around the canaliculi possessed degenerated cristae (Figs 6, 8). Chief cells' nuclei represented enlarged perinuclear spaces (Fig. 7).

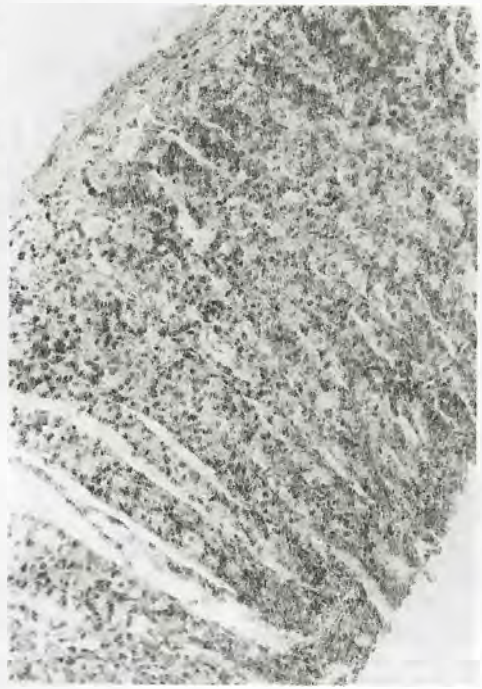

Fig. 4. Light microscopy of the stress group. H. E. stain, ×33

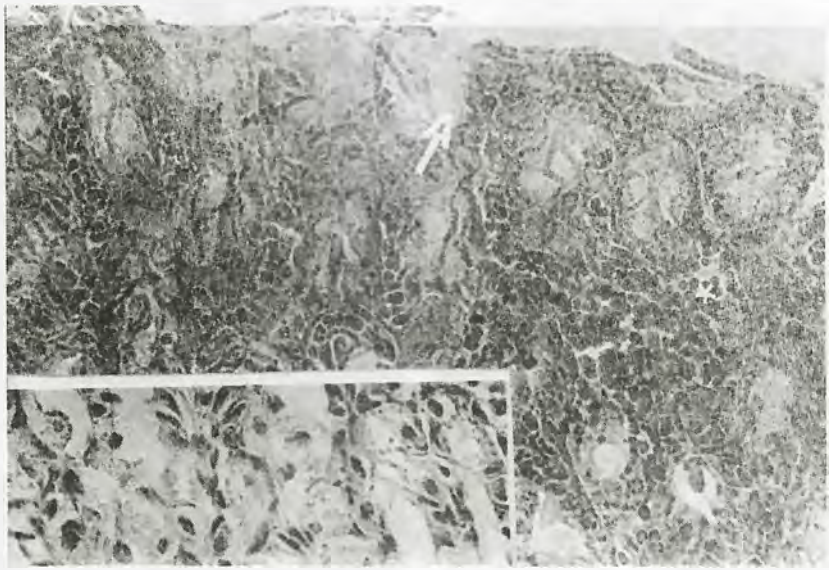

Fig. 5. Stress group light micrograph indicates disrupted surface mucous cells (arrow). Distinct free erythrocytes at the mucosa are seen. Inset: vacuolated glandular cells. H. E. stain, ×132

Fig. 6. Stress group at TEM level shows pyknotic nucleus (nu), mitochondrial crista damage (*), enlarged intracellular secretory canaliculi (arrow) at a parietal cell
ec — endocrine cell

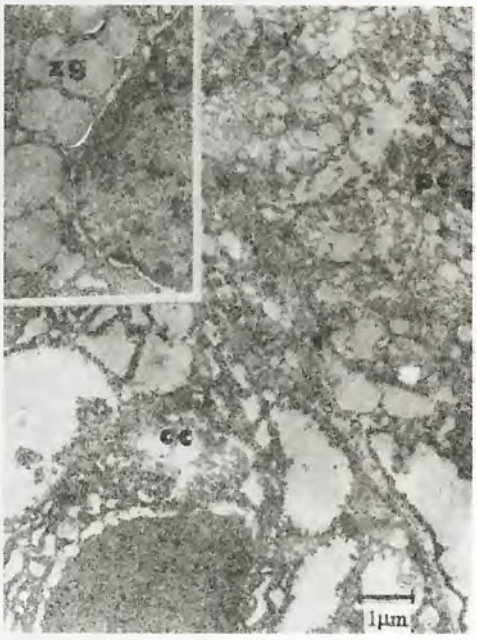

Fig. 7. TEM observation of the stress group: cellular damage at the parietal cells (pc) and chief cells (cc) is observed. Inset: zymogenic granules (zg) at a chief cell

Fig. 8. Stress group electronmicrograph: widened intracellular secretory canaliculi (*) and mitochondrial degeneration at a parietal cell

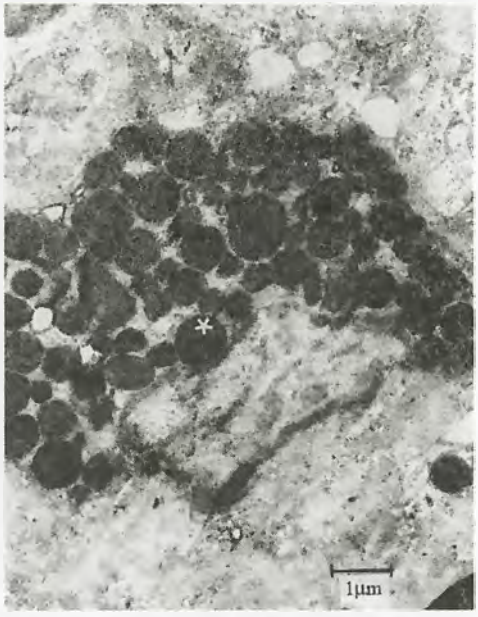

Fig. 9. Mast cell with numerous secretory granules (*) is observed at the stress group electronmicrograph

Fig. 10. Periphery of the ulcer zone; surface mucous cells were flattened with irregular cell borders. Wide openings to the gastric pits (*), few erythrocytes, necrotic area (arrow)

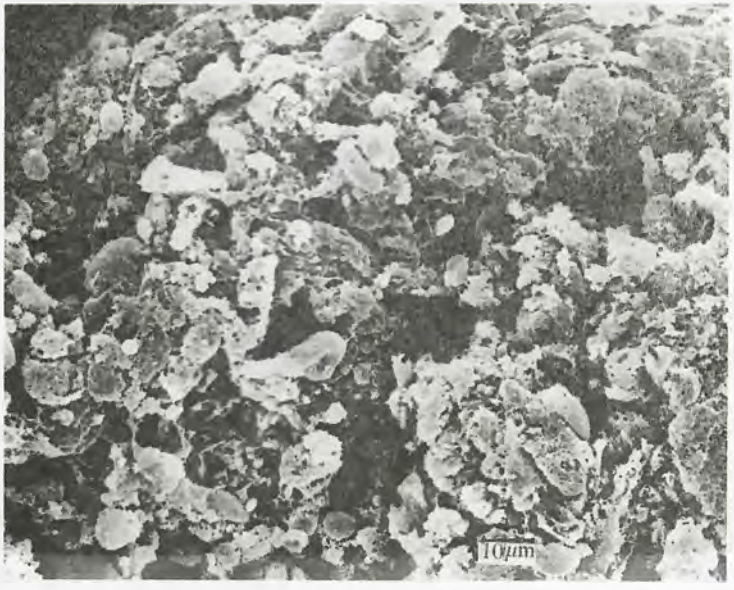

Fig. 11. Ulcer area; remnants of degenerated epithelial cells. Gastric pits (*) are widened and devoid of epithelium

Fig. 12. Hemorrhage area; Erythrocytes with distorted morpohology. Characteristic echinocytes (arrow) are present

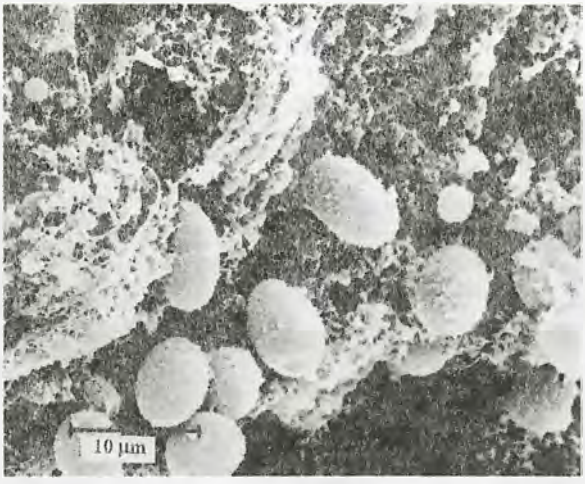

Fig. 13. Individual epithelial cells without cellular junctions. A characteristic fibrin coat (arrow) is present

Extremely dilated endoplasmic reticulum membranes reflected a high degree of cell damage. Abundant secretory granules filled the apical cell cytoplasm (Fig. 7). Endocrine cells were observed with their secretory granules. Their organelles did not reflect a cellular damage (Fig. 6). Mast cells, as a connective tissue component, were noticed with numerous granules which seemed to be increased (Fig. 9).

Scanning electronmicroscopical investigations revealed that:

a) In region with a normal appearance close to the ulcer area, flat epithelial cells and loose cellular interaction were observed. Few scattered erythrocytes were dispersed (Fig. 10).

b) In ulcer area, a totally necrotic appearance was dominant. Epithelial cells were no longer observed individually. Only degenerated remnants of cells were present. Gastric pits were observed widened and devoid of lining epithelium (Fig. 11). In the centre of ulcer, a hemorrhagic area was noticed with numerous erythrocytes of abnormal morphology (echinocytes) as a sign of extravasation (Fig. 12). A characteristic network of fibrin coat and individual epithelial cells without cellular junctions were also determined at the SEM observations of the ulcer group (Fig. 13).

Discussion

According to some investigators, stress ulcer formation is primarily mediated by a marked increase in gastric muscular contractility and ischemia [4, 6]. Parallel to the ulcer formation, both an increase in gastric lipid peroxidation level and a decrease in glutathione level were described [7]. Investigators mentioned the high degree of gastric mucosa cellular degeneration and related it to increased levels of lipid peroxidation as a result of stress application [7, 8, 9]. Calcium was also mentioned as an important point in the ulcer formation in which increased gastric muscle Ca^{++} levels were correlated with the magnitude of gastric lesions observed [10, 11].

We define the parietal and chief cells as the most affected cells of the stress group. Extremely widened intracellular secretory canaliculi of the parietal cells implied an extreme HCl secretion (Figs 6, 7, 8). While those canaliculi were swollen, tubulovesicular structures around them were restricted (Figs 7, 8). That morphological appearance was interpreted as a membrane transport form tubulovesicular structures toward the widened intracellular secretory canaliculi. In literature, that reverse correlatoin between them was mentioned [12]. Chief cells of the stress group were noticed with extremely dilated granular endoplasmic reticulum membranes. Apical secretory granules reflected their storage phase (Fig. 7). Those findings were correlated with literature mentioning an obvious increase in pepsinogen synthesis at the acidic medium. It could be explained that extreme HCl secretion by parietal cells might induce pepsinogen synthesis of the chief cells [13].

Some authors suggested that histamine release from mast cells could induce HCl secretion [9, 13, 14]. The mast cells with numerous granules at the stress group could support that suggestion (Fig. 9).

Hemorrhagic regions observed at light and scanning electron microscopical level were distinct especially at the upper gastic mucosa (Figs 4, 5, 10, 12). Those observations were parallel to the literature [15, 16]. We want to correlate that findings to the cellular damage observed at TEM level (Figs 6, 7, 8). A delay in vascular supply due to the hemorrhage could cause an ischemia and that may provoke the mentioned cellular degeneration.

As a conclusion, individual findings at the level of light, transmission and scanning electron microscopy were parallel and showed correlation with the physiologic and biochemical approaches of the experimental stress ulcer.

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CSA production in murine long term bone marrow cultures after in vitro treatment with ranopterins[1]

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The presence of colony stimulating activities (CSAs) in the supernatants of murine long term bone marrow cultures (MLTBMCs) was analysed after treatment with two pteridine fractions (ranopterins — Rpts) — pterin-6-carboxyllic acid and pterin. CSAs were measured by the production of colonies and clusters from macrophage-granulocytic (MG-) cell lines in a semi-solid medium (murine bone marrow agar cultures). The results show a well expressed macrophage-granulocytic colony stimulating activity (MG-CSA) in the supernatants, after the treatment with the pterin-6-carboxyllic acid. This finding supports the hypothesis [1] that ranopterins induce GM-CSA not only directly — in agar cultures, but also indirectly — via CSAs production by stromal macrophages in the MLTBMCs.

Key words: mouse bone marrow,long term cultures, ranopterins, pterin, pterin-6-carboxyllic acid, CSA, MG-CSF.

We have previously reported that "in vitro" treatment with some ranopterins stimulate the macrophageal-granulocytic colony formation (MG-CFU) in murine bone marrow agar cultures [1, 2].

In order to determine whether the haemopoietic stimulation of macrophage-granulocyte (MG-) cell line induced with ranopterines was mediated only directly — by the effect of the biologically active substances on earlier progenitors [1], or indirectly — by the release of colony stimulating factors (CSFs), we analysed the presence of colony stimulating activities (CSAs) in the supernatants of long term bone marrow cultures (LTBMCs) after treatment by different doses of ranopterins.

[1] Part of the work was presented in the International Congress:
E. Zvetkova, E. Katzarova, E. Ianeva, B. Nikolov, I. Tsenov. Testing of MG-CSA in murine long term bone marrow cultures after in vitro treatment with ranopterins. — In: Alpenländisches Anatomentreffen, 2-4 Sept., 1994, Pilsen, Czech Republic.

Material and methods

LTBMCs were stabilized according to the technique described by C o u t i n h o et al. [3]. Two weeks after the stabilization, different doses of ranopterins — pterin and pterin 6-carboxyllic acid (10, 25 and 50 µg/ml culture) were added, and seven days later the supernatants were collected.

Measurements of CSAs in the supernatants were performed by assessing of MG-CFU colony formation where murine bone marrow cells were cultured in semi-solid media (agar cultures), in the presence of different concentrations of supernatans. As a positive control for the colony stimulating activity the conditioned medium from the 3T3 cell line was used [4]. As a negative control the conditioned medium from LTBMCs without addition of ranopterins was applied.

The supernatants of LTBMCs treated with two ranopterins, induced the formation of macrophageal-granulocytes (MG-) and/ or macrophageal (M-) colonies in the murine bone marrow agar cultures, cytologically characterized after in situ staining with methylene blue and fast green [5], by fluorochromation in situ for basic cytoplasmic proteins [6] as well as by the methods of SEM and TEM electron microscopy (applied for the cells present in colonies).

Results and discussion

The results from the study show a well-expressed macrophageal-granulocyte colony-stimulating effect after the treatment with one of the ranopterin fractions — Pterin 6-carboxyllic acid. The number of CFU-MG depends also on the concentration of the supernatant added, as well as on the dose of the ranopterins administered to the LTBMC. The CSA production is higher when the higher dose (50 µg/ml LTBMC) of Pterin 6-carboxyllic acid was added. In contrast, no MG-CFU formation occurs when

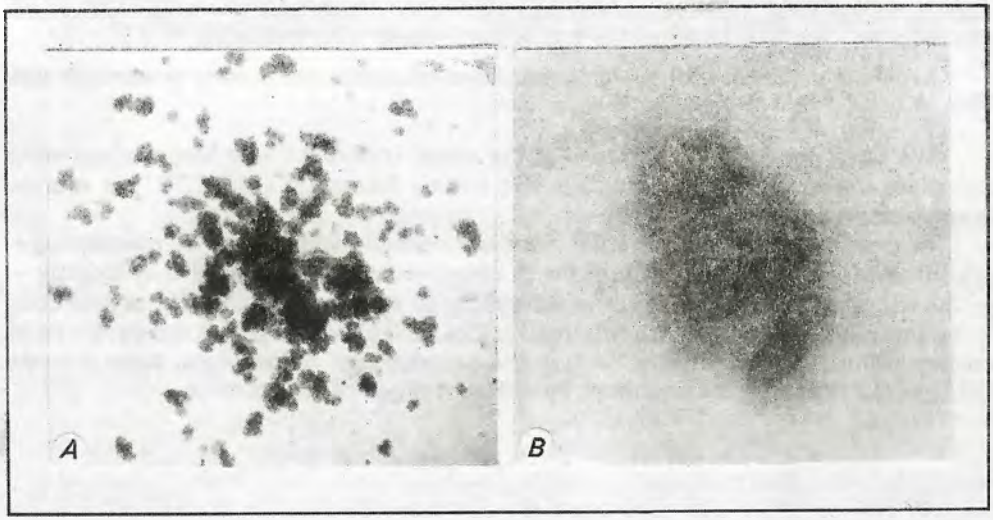

Fig. 1. Macrophageal-granulocyte (MG-) colony from murine bone marrow agar culture, after a 7 day culturing

A — MG-colony, × 350; *B* — monocytes and macrophages from the same colony, × 1000, Immersion

supernatants from control LTBMCs were added to agar cultures, even when concentrated supernatants were applied.

The cytological and cytochemical study of different cell groups and the cell types included in colonies and clusters shows a different degree of macrophageal maturation with the prevalence of immature cells and small macrophages in some of them (Fig. 1). The latest are probably "active" in the synthesis and secretion of a great variety of compounds and biological activities, the tumour-necrotic factors (TNF) including, which has been confirmed for some similar small- and medium-sized macrophageal populations of multiple location [7].

The results showed that some ranopterins induce, in a dose-dependent manner, the production of CSA in LTBMCs which supports our hypothesis that some ranopterins induce hemopoietic (MG-colony stimulating activity) not only directly, but also via CSF-s production in LTBMCs. The results of our experiments in vitro correlate also with data [8], establishing that some ranopterins increase the activity of hemopoietic organs in vivo.

Acknowledgements

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Attempts to localize histochemically aspartylglucosaminidase

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A direct tetrazolium salts method for the histochemical localization of Aspartylglucosaminidase (AGA) is proposed. The method is based on the substrate *L*-aspartic acid-β-menadiol ester. The precise localization of the enzyme in different rat organs can be revealed by the new method. For the first time AGA is localized histochemically in tissue sections.

Key words: Aspartylglucosaminidase, Menadiol, Tetrazolium salts method, Enzyme histochemistry.

Introduction

N-glycoproteins represent complex biochemical compounds, in which the oligosaccharide chains are bound to the protein by an amide bond between 1-amino-N-acetylglucosamine and the β-carboxyl group of *L*-aspartic acid. In animal cells this amide bond is hydrolysed by N-aspartyl-β-N-acetylglucosamineamidohydrolase (aspartylglucosaminidase, AGA, EC 3.5.1.26), a specific enzyme of the amidohydrolase group. The enzyme acts only on substrates with a single aspartic acid residue, i. e., after the proteolytic digestion of the protein chain. AGA has been isolated and purified from different human and animal organs, e. g. from rat [1,2] and human [3, 4, 5] liver and its biochemical properties have been studied extensively. The genetically determined deficiency of the enzyme, i. e. the aspartylglucosaminuria disease results in the secretion of uncleaved aspartylglucosamines into the urine. Using polyclonal antibodies AGA has been localized in the lysosomes of the fibroblasts in healthy humans, but has not been found in patients, suffering from aspartylglucosaminuria [6]. Recent investigations on AGA show, that the enzyme can act not only as an amidohydrolase but as an esterase as well. For example, AGA hydrolyses the β-methylester of Asp [7]. This property of the enzyme has prompted us to synthesize the β-esters of Asp with 1- and 2-naphthols and menadiol and to use these compounds as substrates for the histochemical localization of AGA. For the same purpose we have synthesized the β-amide of Asp with 1-naphtylamine.

Materials and Methods

1. *Synthesis of the substrates.* The synthesis of the substrates was carried out using Z-Asp-α-benzylester and 1-naphthol, 2-naphthol, menadiol or 1-naphthylamine in tetrahydrofuran with dicyclohexylcarbodiimide. After the purification of the substances obtained during the reaction, the protective groups of Asp were cleaved with H_2/Pt on activated carbon.

The specific inhibitor of AGA, 5-diazo-4-oxo-L-norvaline (DONV), was synthesized according to H a n d s c h u m a c h e r et al. [8].

2. *Animals.* Wistar rats of both sexes were used. After decapitation in deep ether anaesthesia, samples from different organs were fixed in 2 % formaldehyde (freshly prepared from paraformaldehyde) in 0,1 M phosphate buffer, pH 7,6 for 24 hours at 4 °C. Then, the pieces were washed for 24 hours in Holt's solution, frozen in liquid nitrogen and 10 μm cryostat sectons were cut.

3. *Incubation solutions.* The cryostat sections were incubated for 1 hour at 37 °C in the following incubation solutions: 1 to 3 mM *L*-aspartic acid-4-(1-naphthyl)-ester (Asp-1-NE) or *L*-aspartic acid-4-(2-naphthyl)-ester (Asp-2-NE) and 1 mg Fast Blue B/ml in 0,1 M phosphate buffer, pH 7,2; 1 to 3 mM *L*-aspartic acid-4-(1-naphthyl)-amide (Asp-NA) and 0,03 ml freshly diazotized New Fuchsine/ml in 0,1 M phosphate buffer, pH 6,5; 1 to 3 mM *L*-aspartic acid-4-monomenadiolester (Asp-ME) and nitro blue tetrazolium (NBT) or tetranitro blue tetrazolium (TNBT) in 0,1 M Tris buffer, pH 7,6. After incubation the sections were post-fixed in 4 % neutral formaldehyde, washed with water and embedded in glycerol-gelatine. Parallel sections were incubated in the same medium in the presence of 200 μM DONV. Additonally, other sections were incubated without the AGA substrates and termed as controls or with menadiol diacetate and NBT for the demonstration of non-specific esterases after D i k o w and G o s s r a u [9] with or without the AGA inhibitor DONV.

Results

The best results were obtained with the substrate Asp-ME, employing the tetrazolium salts method. The reaction products, i. e., the diformazans of NBT or TNBT, were localized as fine granules in the epididymal principle and clear cells as well as in spermatozoa (Fig. 1), hepatocytes (Fig. 2), epithelial cells of the proximal renal tubules (Fig. 3), macrophages and reticular cells of the spleen, enterocytes of the intestine (Fin. 4) and in giant lysosomes of the ileum of 7-day-old rats (Fig. 5). In the presence of the AGA inhibitor DONV the enzyme activity was almost completely suppressed in the epididymis, liver, kidney and spleen but in the intestine of suckling and adult rats AGA activity was unchanged.

Using Asp-1-NE or Asp-2-NE as substrates, the azo-dye showed the same localization of AGA as with Asp-ME, however the contrast was considerably lower. No reaction product was observed when Asp-NA was used as a substrate. The demonstration of non-specific esterases with menadiol diacetate and NBT showed a similar localization in the studied organs as with the AGA substrates, but the enzyme activity did not change in the presence of DONV.

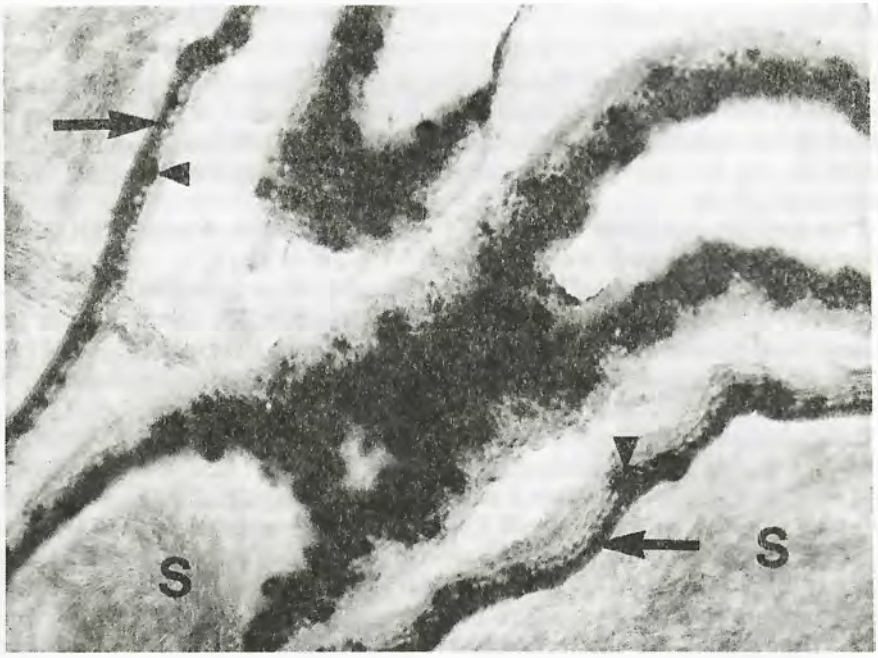

Fig. 1. Rat epididymis. Demonstration of AGA with Asp-ME as a substrate and NBT. The granulated reaction product — NBT diformazan appears in the principle (arrows) and clear (arrowheads) cells and in spermatozoa (S). × 400

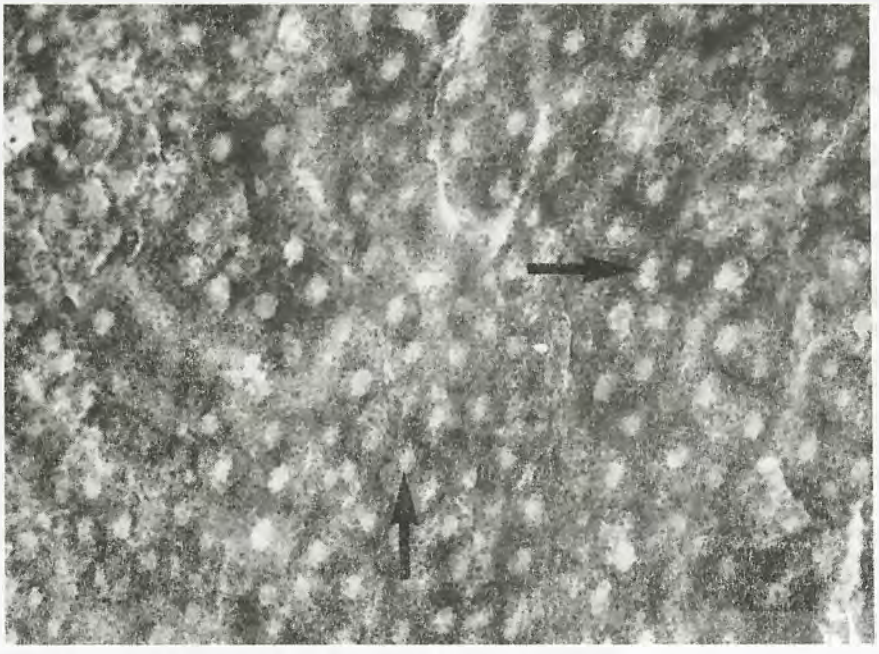

Fig. 2. Rat liver. Demonstration of AGA activity with Asp-ME and NBT. The reaction product — NBT diformazan is present in hepatocytes (arrows). × 400

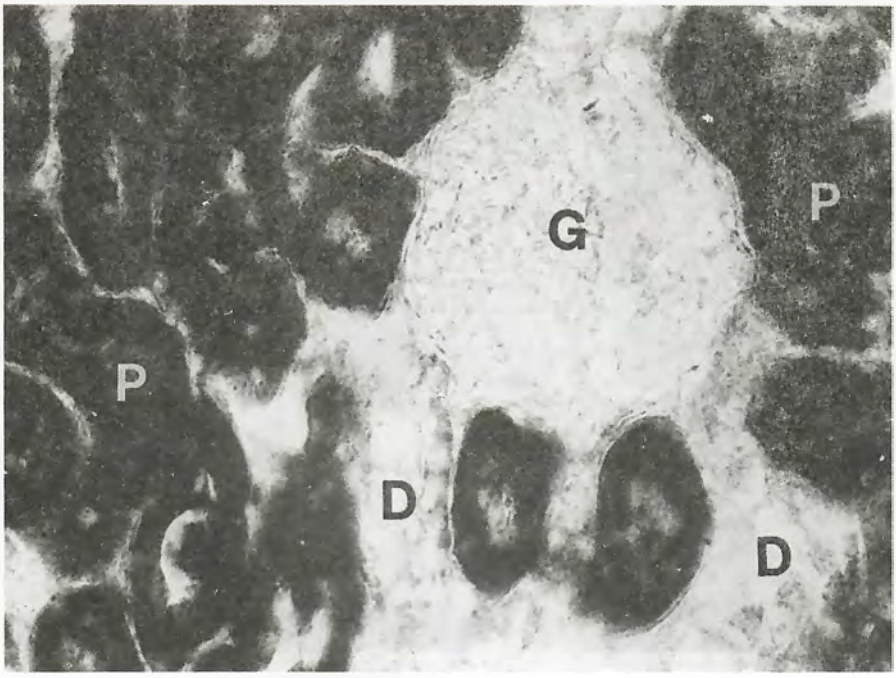

Fig. 3. Rat kidney. Demonstration of AGA with Asp-ME and TNBT. The reaction product – TNBT diformazan is visible in the epithelial cells of the proximal renal tubules (P). ×400
G – glomerulus; *D* – distal tubules

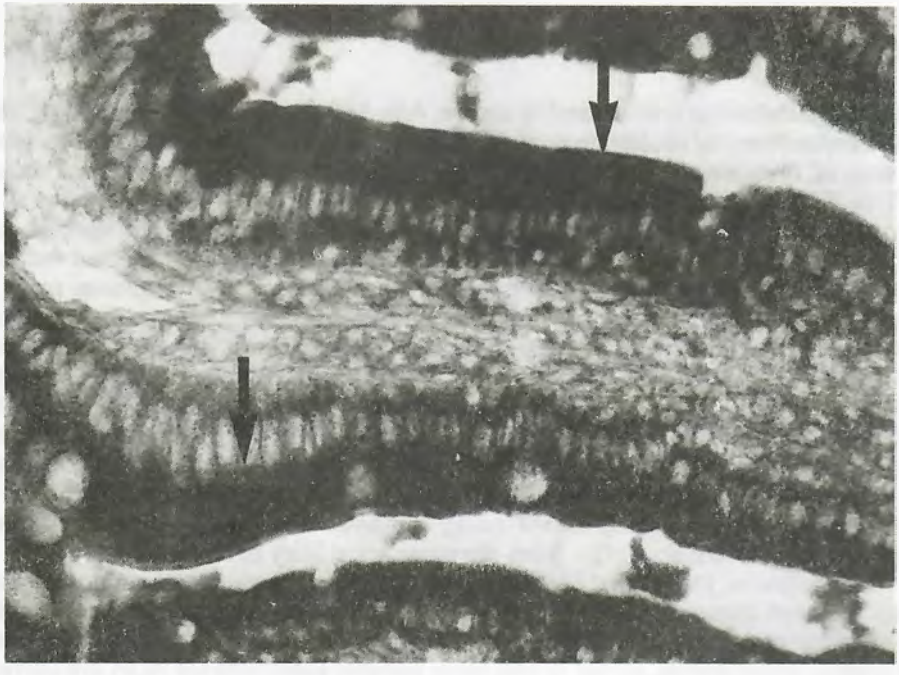

Fig. 4. Rat intestine. AGA demonstration with Asp-ME and NBT. High amounts of NBT diformazan are seen in the enterocytes (arrows). ×400

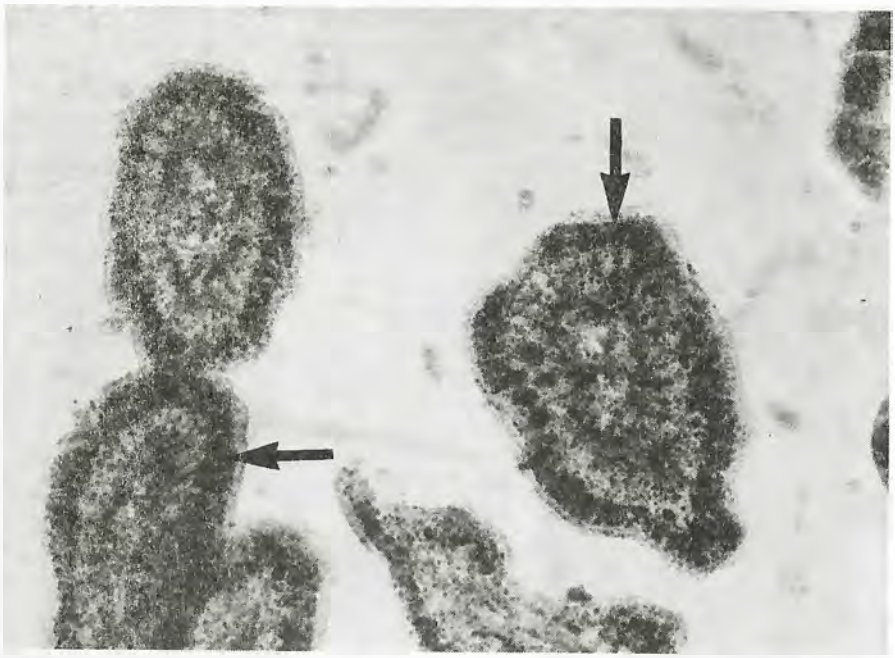

Fig. 5. Ileum of a 7-day-old rat. Visualization of AGA activity with Asp-ME and TNBT. TNBT diformazan is present in the region of giant lysosomes (arrows) of the enterocytes. ×400

Discussion

One of the substances synthesized by us, i. e. Asp-ME appeared to be a good substrate for the histochemical localization of AGA. This compound was readily hydrolysed at the sites of lysosomes in many cells of rat tissues; the liberated menadiol reduced NBT or TNBT to intensively coloured diformazans, thus marking the sites of the enzyme activity. The staining was almost completely inhibited by the specific AGA inhibitor DONV in most of the studied organs, but no inhibition was observed in the rat intestine. The histochemical demonstration of non-specific esterases using the same tetrazolium salts procedure, but with a different substrate (menadiol diacetate) showed co-localization with AGA and non-specific esterase activity was not suppressed by the AGA inhibitor DONV. These data sugest, that the enzyme activity, remaining after AGA inhibition by DONV could be ascribed to non-specific esterases, which partially also hydrolysed Asp-ME. Therefore, when Asp-ME is used as a substrate for the histochemical localization of AGA, the co-reaction of non-specific esterases has always to be considered and gives the method only a limited specificity. On the other hand, all biochemical assays using synthetic substrates, e. g. aspartic acid amidomethylcoumarines suffer from the same specificity problems.

In conclusion, for the first time an enzyme of the N-glycoproteinamidohydrolases group has been demonstrated histochemically, which provides us with the possibility to obtain more information about AGA in heterogenously structured cells, tissues and organs. Experiments, aimed to find more suitable substrates for AGA are currently in progress in our laboratories.

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Histochemical localization of alanineaminopeptidase by means of a new substrate — dialanine-1,4-phenylenediamide with the tetrazolium salts method

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A new substrate, based on 1,4-naphthylenediamine has been synthesized and used for the histochemical demonstration of Alanine aminopeptidase (APM) according to the tetrazolium salts method. The new substrate reveals the correct localization of the enzyme concerned. The histochemical localization of APM in tissue sections from different rat organs is presented.

Key words. alanineaminopeptidase, dipeptidyl peptidase IV, tetrazolium salts method, enzyme histochemistry.

Introduction

Alanineaminopeptidase (APM, EC 3.4.11.2) is a membrane protease, which cleaves preferably *L*-Alanine (*L*-Ala) from the N-terminal of the peptides. The enzyme is widely spread in human and animal organs and tissues, but mostly in kidney, small intestine, bile capillaries, capillary endothelium and epithelial cells of different organs.

Hitherto, the histochemical localization of APM has been performed using the amides of *L*-Ala with 1- and 2-naphthylamines or 4-methoxy-2-naphthylamine as substrates. The arylamines, liberated from the enzyme hydrolysis couple with diazonium salts and the azo-dyes, obtained during this reaction, mark the sites of the enzyme activity [1]. Attempts at the visualization of APM by means of an indigogenic substrate — *L*-N-(5-bromo-indol-3-yl)-leucinamide hydrochloride has been made by several authors [2,3]. According to this method, if tetrazolium salts (TNBT or NBT) are used as oxidating agents, two different colourful substances i. e. the bisindigo and the respective formazan precipitate at the sites with a high enzymatic activity.

A new substrate for the histochemical localization of APM — *L*-Ala-5-chloro-1-anthrachinoylhydrazide has been synthesized in our laboratory [4]. The enzyme hydrolysis liberates a deeply coloured insoluble product — 5-chloro-1-anthrachinoylhydrazine, which can be converted into 4-dimethylaminobenzylidene anthrachinoyl-1-hydrazone, which increases considerably the contrast. In the present paper we are considering the possibilities for the histochemical localization of APM with a new substrate, synthesized by us — dialanine-1,4-phenylenediamide in combination with the tetrazolium salts procedure.

Materials and Methods

Synthesis of the substrate

The synthesis of the substrate was carried out according to the acid chloride procedure [5]. The acid chloride of Z-Ala was obtained with phosphorus pentachloride and it was coupled with 1,4-phenylenediamine dihydrochloride in the presence of Na_2CO_3. The compound, obtained according to this procedure was washed with 1 N HCl, water, saturated aqueous solution of $NaHCO_3$, water and dried out. After that, the protective group of the amino acid was cleaved with hydrobromic acid in acetic acid.

The monotetrazolium salt 2,3,5-tri-(4-nitrophenyl) tetrazolium chloride (TNPT) was synthesized by us after Seidler [6].

Substrate solutions

The histochemical localization of the enzyme was performed on 10 µm thick cryostat sections from Wistar rat organs, non-fixed or fixed in acetone — chloroform mixture (1:1) for 5 min at —20 °C. The substrate solutions consisted of 1 mM *L*-Ala-1,4-phenylenediamide dihydrobromide and 1 mg/ml BSPT (2-(2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl) tetrazolium chloride), TNPT (2,3,5-tri-(4-nitrophenyl) tetrazolium chloride) or NBT (nitro blue tetrazolium chloride), dissolved in Tris buffer 0,1 M with a final pH value of 7,2. After one hour incubation at 37 °C the sections were post-fixed in 4 % neutral formaline and embedded in glicerine-gelatine.

Results

After the enzyme hydrolysis of the substrate, the liberated 1,4-phenylenediamine reduced the available tetrazolium salt to a formazan, which precipitated on the places with the enzyme activity. The best results were obtained on the fixed sections with the monotetrazolium salt TNPT, which was due to its low redox potential. Similarly good results were obtained with BSPT, but in this case the localization was less precise. In the studied organs APM was localized in the brush borders of the epithelial cells of the proximal renal tubules, but in the glomerulus the reaction product was not observed (Fig. 1). In the intestine the monoformazan precipitates were seen in the brush borders of the enterocytes and in the macrophages of the lamina propria (Fig. 2).

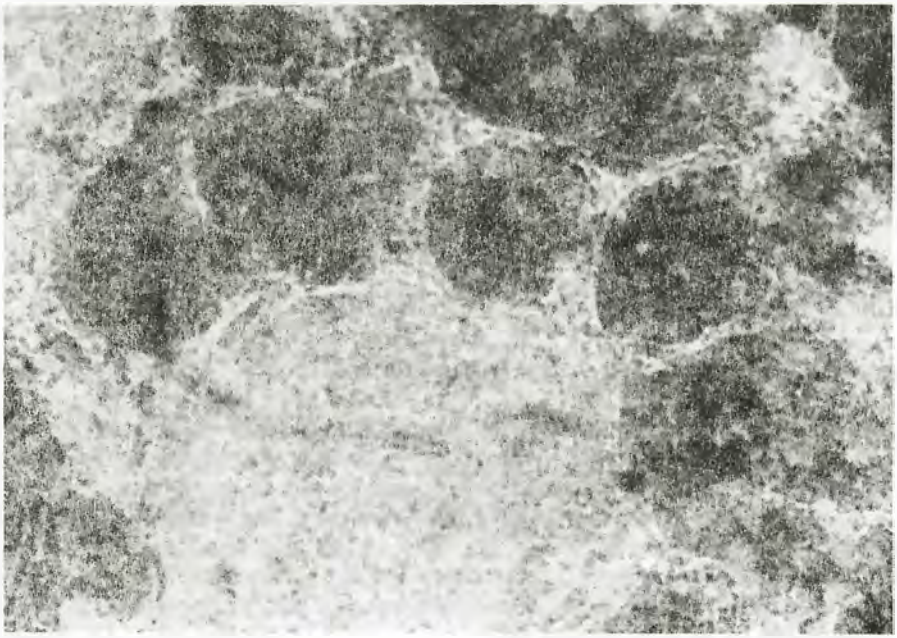

Fig. 1. Rat kidney. Histochemical localization of alanineaminopeptidase with dialanine-1,4-phenylenediamide and TNPT. The formazan precipitates are seen in the brush borders of the epithelial cells of the proximal renal tubules. ×400

Fig. 2. Rat intestine. Histochemical demonstration of APM with dialanine-1,4-phenylenediamide and TNPT. The monoformazan is precipitated in the brush borders of enterocytes and in the macrophages of the lamina propria. ×400

Discussion

The different methods for the histochemical localization of alanineaminopeptidase, used hitherto, suffer from many disadvantages. For example, the method with the substituted and non-substituted naphthylamine amides of *L*-Ala requires the pH value of the incubation medium to be maintained at about 6,5, because otherwise the reaction of coupling between the naphthylamine, liberated during the enzyme hydrolysis and the diazonium salt is not possible. On the other hand, the diazonium salts themselves are widely known as inhibitors of the enzyme activity [7]. The method with the indigogenic substrate *L*-N-(5-bromo-indol-3-yl)-leucinamide hydrochloride [2] without any oxidation agent in the substrate solution has the major drawback — the prolonged incubation, which causes the considerable difusion of the reaction product. The same procedure with the use of tetrazolium salts as oxidation agents [3] leads to two final colourful reaction products (an indigoid dye and a formazan), which makes it impossible to perform any quantitative studies.

The substrate for the histochemical localization of APM — dialanine-1,4-phenylene diamide, synthesized by us, localizes precisely the enzyme activity, when used in conjunction with the tetrazolium salts procedure. It allows the pH value of the substrare solution to be kept at the optimal level for the enzyme, because the tetrazolium salts reduction goes easily in a broad range of pH. On the other hand, the reaction product — the formazan of the given tetrazolium salt is the only colourful substance, obtained at the end of incubation, thus permitting to perform quantitative histochemical investigations on the studied enzyme.

It is important to mention here that the new method described in this communication allows the histochemical localization of many amidohydrolases, if the appropriate amino acid is bound to 1,4-phenylenediamine through an amide bond.

As a prospect of future, we intend to synthesize the diamides of different amino acids with 1,4-diaminonaphthalene and use them as substrates for the histochemical localization of the respective peptidases with the tetrazolium salts method, because this compound (1,4-diaminonaphthalene) is an even more powerful reducing agent than 1,4-phenylenediamine.

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Anthropology

Anthropometrical characteristics of the nose of adult Bulgarians

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The aim of the present paper is to make a detailed metrical characteristics of the nose in adults Bulgarians from both sexes and to study the specificity of the intersexual metric differences. 100 men and 103 women at the age of 21 up to 40 year are invesigated. The study's programme consist of 13 metric features (6 of which are newly introduced from the authors) and 2 indices — one characterizing the nose form and the other — the intersexual differentiation of the studied features.

The results show that the category — leptorrhin dominate in the investigated men and women. Juxtaposing the height and length of the nose it is obvious that the top nose position defines a transition form between concave and straight ones. The intersexual differences in the metrical data of all the investigated features are statistically significant — except the radix nose breadth. The other nose breadths are with the highest sexual differentiation.

Key words: anthropometry, human nose, metrical variety, intersexual differences.

Anthropometric characterization of the particular body organs and parts is both of anthropological and of medico-biological importance as well as it is valid for application in the practice. In this respect the study of the shape and the size of the nose and its prominence in particular which represent some of the most specific for the human somatic features, renders a special scientific interest.

Anatomic and anthropological sciences dispose of various and exact data about nose structure and its function as an olfactory and respiratory organ, about its growth and development in phylogenesis as well as in ontogenesis [1, 2, 3, 6, 7]. It is a pity, however, that the anthropological data on the normal metric variety of the morphological characteristics of the nose, are scanty although they are of especially great importance both in the sense of general biology as criteria for age differences, sexual differentiation and so on, and for the diagnosis and treatment of various inborn and acquired defects and malformations of the nose. In medico-biological literature scarce data is found most often about cases with morphological pathology of the nose and about concrete operative approach of the rhinoplastics carried out. Purely anthropologically metric data on the nose are published mainly as part of the total

cephalometric characteristics of the man in which most often only the length, height and width of the nose are included. Representative cephalometric studies have been carried out in Bulgaria with data published about all three basic parameters by St. Vatev, M. Popov, Y. Yordanov etc. [4, 5, 8]. In the purposefully surveyed literature, however, a special attention should be paid to the writings of Madjarov et al. revealing data about a great number of metric characteristics of the nose and its distances from the chin, the mouth, and the ears without analyzing, however, the specificity of the intersexual differences among them.

The AIM of the present study is to work out detailed metric characteristics of the nose in adult Bulgarians from both sexes and to study the specificity of the intersexual metric differences.

Material and methods

Detailed cephalometric study has been carried out including 13 metric features of the nose in 100 men and 103 women aged between 21 and 40 (Figs. 1, 2). All individuals studied were clinically healthy and with preserved dentition and occlusion. Six of the metric features of the nose were introduced by us for a more detailed characterization of the shape and size of the allae nasi, the nostrils and the outer septum between them (No. No. *6–13* of the Table 1). We additionally calculated two indices as well one of which typified the nose shape and the other characterized the sexual differentiation according to the different features. The metric data have been biostatistically processed and the statistical significance of the intersexual differences was estimated by the Student's t-criterion at a significance level of 0,05. The degree of sexual defferentiation has also been determined for the different features the

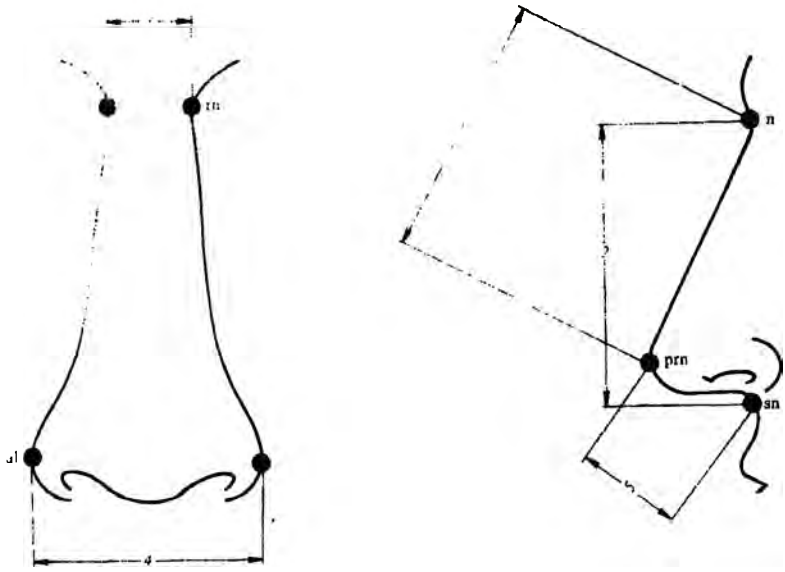

Fig. 1

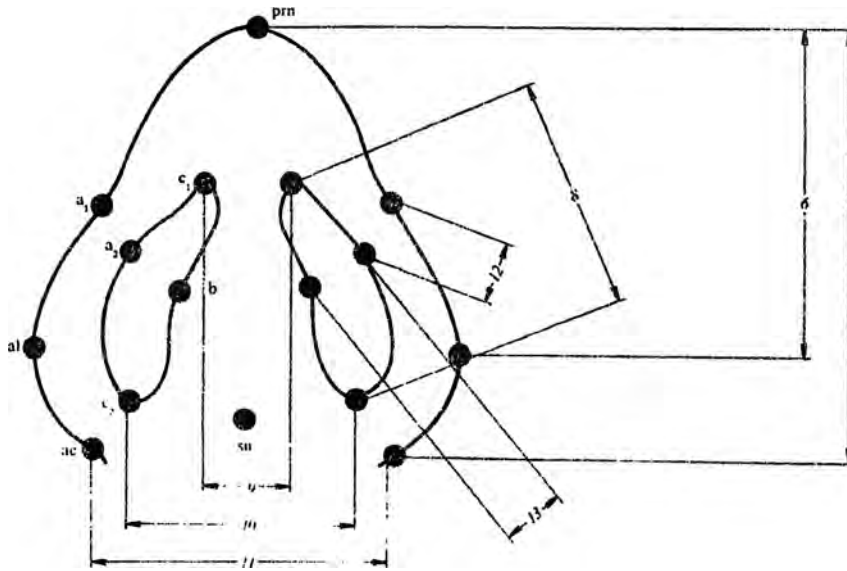

Fig. 2

gradation being carried out by the percentile analysis on the basis of the value range of the corresponding indexes. In the data from the present investigation the range is 16,67. Three degrees of sexual differentiation have been distinguished. The values limiting them are as follows: I degree — 100—105,55; II degree — 105,56—111,10; III degree — 111,11—116,67.

Results, analysis and discussion

The metric data are shown in table 1.

A general aspect of the nose shape is presented by the index of the ratio between the nose width and nose height. According to its categories after Martin and Saller (Fig. 3) among the individials studied by us the leptorrhin ones prevail—in women the account for even more of 90 per cent of the cases. In the category of the mesorrhin ones 18,56 per cent of the males and only 6,80 per cent of the women are represented. In the chamerrhin category none of the studied males are present and in women it is found extremely rarely — in only 0,97 per cent of the cases. These results show that in the individuals of our sample the proportion of the nose as a whole is elongated this trait being better pronounced in women. Similar are the data about the nose shape of other investigators of the Bulgarian population such as St. Vatev and M. Popov [5, 7]. Having in mind that their studies have been carried out on a much great number of individuals i. e. their sample is much more representative it follows that the identical result about this fundamental nasal characteristics confirms indirectly the representativity of the data from our study also about the rest of the investigated features.

An additional idea of the nose shape is also derived from the juxtaposition between the values of the two basic dimensions of the nose — its height and its length. Both in men and women the two features have relatively identical values with a slight

Table 1. Investigated features

No — features	Men			Women			T	σ/♀
	$\bar{x}$	σ	v	$\bar{x}$	σ	v		%
1 — nose length (n—prn)	5,52	0,40	6,69	5,12	0,39	7,56	7,27	107,81
2 — nose height (n—sn)	5,60	0,34	6,12	5,26	0,35	6,66	6,94	106,46
3 — radix nasi breadth (rn—rn)	1,93	0,23	12,02	1,90	0,25	13,09	0,88	101,58
4 — nose breadth (al—al)	3,66	0,25	6,70	3,24	0,26	7,80	11,67	112,96
5 — nose protrusion (prn—sn)	2,04	0,23	11,13	1,97	0,25	12,80	2,06	103,55
6 — ala nasi length to the alare (prn—al)	3,29	0,20	6,11	2,97	0,24	7,98	10,32	110,77
7 — ala nasi length to the nose base (prn—ac)	3,05	0,22	7,27	2,79	0,22	7,99	8,39	109,32
8 — longitudinal diameter of the naris ($c_1 - c_2$)	1,66	0,20	12,31	1,46	0,19	13,32	7,14	113,70
9 — septum nasi breadth at the front ($c_1 - c_1$)	0,73	0,13	17,48	0,66	0,12	18,00	3,89	110,61
10 — septum nasi breadth at the back ($c_2 - c_2$)	2,17	0,24	10,97	1,86	0,21	11,36	9,69	116,69
11 — breadth of nose base (ac—ac)	2,38	0,28	11,56	2,11	0,21	10,14	7,71	112,80
12 — ala nasi thickness ($a_1 - a_2$)	0,53	0,09	16,42	0,48	0,09	18,79	3,85	110,42
13 — transversal diameter of the naris ($a_2 - b$)	0,83	0,13	16,05	0,77	0,12	15,09	3,33	107,79
14 — index of nose form (al — al) : (n—sn)	65,62	5,67	8,64	61,75	6,52	10,55		

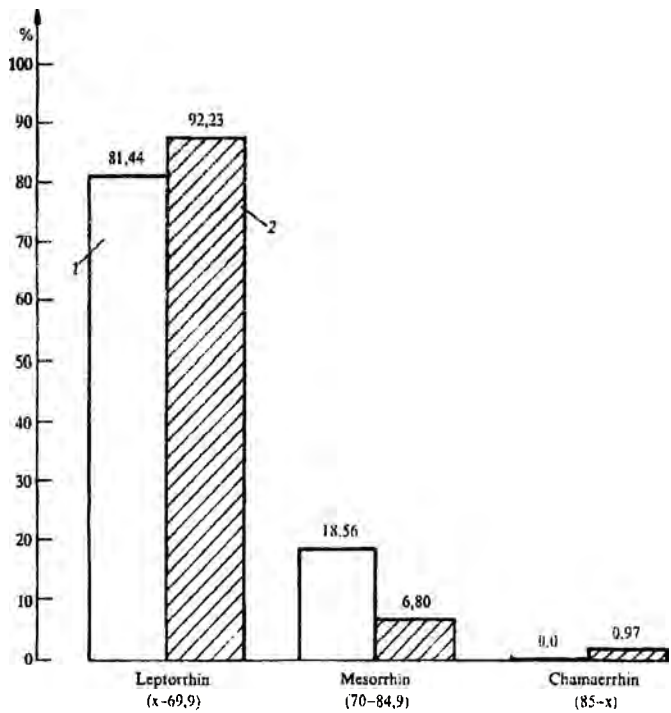

Fig. 3. Nose form — categories by Martin and Saller [3] and its percentage distribution

1 — males; *2* — females

prevalence of the height size. These data show that the position of the nose apex as opposed to its basis rates it a nose of a transitional shape between the concave and straight ones.

The morphograms constructed render a more wholesome picture of the metrical characteristics of the nose of the individuals under study (Fig. 4). The numerical signs for the different radiants correspond to the original numbers of the features in the table. As it is seen from the morphograms with the whole set of the studied features the males are logically with bigger sized noses. Where as it must be noted that the metrical intersexual differences are statistically significant for all features with the exception of the radix nasi breadth only. Comparatively speaking, the intersexual differences in the basic lengths and widths of the nose (*1, 2, 4* and *11* features), in the allae nasi sizes (features *6* and *7*) and in the outer sizes of the nose septum (features *9* and *10)* are of greater distinction.

The calculated indexes of sexual differentiation and the corresponding gradation of the established differences give an objectivized and more complete

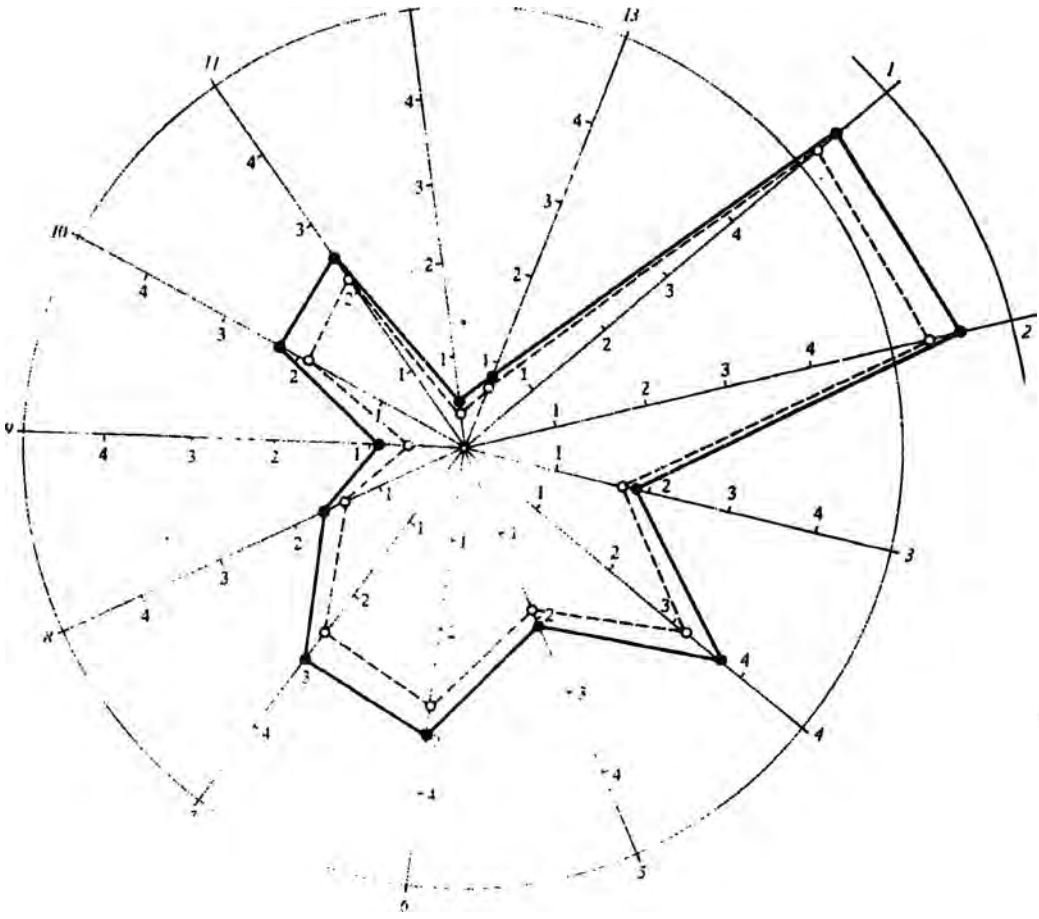

Fig. 4. Morphograms according to metrical data of the investigated features
– males; · · · females; *1* – *13* – features

demonstration of the intersexual differences in the sizes of the features under study (Fig. 5).

As it is seen in the graph the highest III degree of sexual differentiation display the four features: the longitudinal diameter of the naris and the three width features as follows: septum nasi breadth at the back which actually represents the back distance between both nares, the nose breadth and the breadth of the nose base. 7 out of all features under study show a medium degree of sexual differentiation, where the length-thickness nose characteristics prevail such as length and height of the nose, the two lengths of the allae nasi, the transverse diameter of the naris, the thickness of the allae nasi, etc. The lowest degree of sexual differentiation is yielded only by 2 of the features under study — these are the nose protrusion and the radix nasi breadth.

The results obtained in the following investigation can be summed up in the following directions:

1. A detailed metric characterization of the nose of adult Bulgarians of both sexes has been carried out which has never been done before in our country. In this way, data on the metric varieties of the morphological' characteristics of the modern Bulgarian nose are supplied.

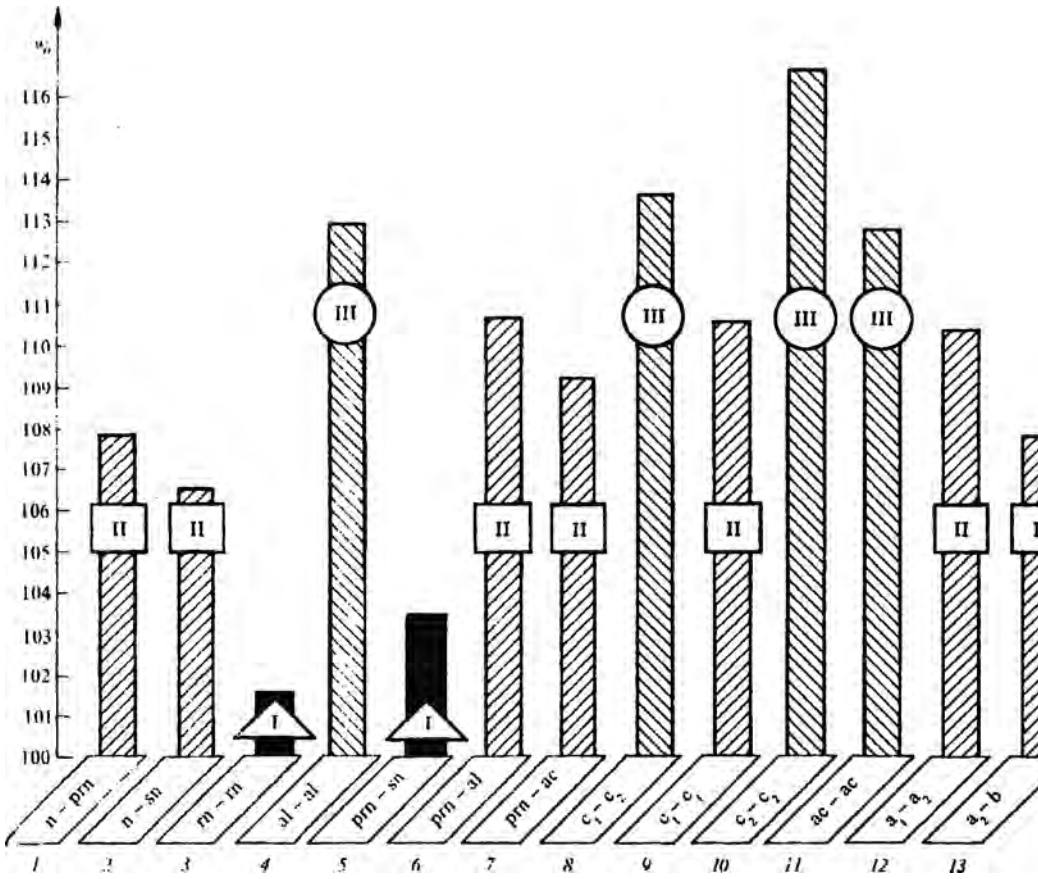

Fig.5. Degrees of the intersexual differences
I – 100–105,55%; II – 105,56 – 111,10%; III – 111,11 – 116,67%; *1 –13* – features

2. The intersexual differences for the features under study are assessed and analyzed establishing that with the exception of the radix nasi breadthall other features show a high degree of statistically significant sexual differentiation.

3. Bearing in mind the fact that there are no such data on the metric characterizartion of nose in the world anthropological literature the complex of features studied in the present investigation can be accepted as a guiding programme for future studies in that direction.

4. Purely pragmatically, the data from the following study have already served as a basis for the workout of phantom models for the production of respiratory protective devices in the military industry — such as breathing masks, etc.

5. In the medico-biological aspect the data obtained can be used in the plastic anthropological reconstruction of the head after the skull and in the practice of rhinoplastic surgery.

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Data about human face asymmetry

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The manifestation of asymmetry in the human face was studied in 130 adult individuals (67 males and 63 females) from the town of Sofia by classic cephalometric methods. The asymmetry was traced in 21 dimensions out of all 28 face dimensions measured bilaterally: 8 of them horisontal, 6 — vertical and 7 — oblique. Ones the data were analyzed biostatistically. Mean values of absolute differences and per cent deviations between the dimensions of the left and right face sides were calculated. In 14 dimensions the percentage of the complete symmetry was higher for the males and in other 7 — for the females.The per cent distribution of the dimensions illustrates approximate symmetry (0±1 mm) following the distribution of the absolute symmetry. Real asymmetry (differences 2 and more in mm) was more frequent in 14 dimensions in women against 7 dimensions — in men.

Key words: anthropometry, facial dimensions, asymmetry.

Asymmetry of the human face is a reflection and part of the bilateral asymmetry in man. The asymmetry in limbs and trunk can in to a certain degree be influenced by environmental factors (profession, sport, habits, etc.) while facial symmetry/asymmetry is predominanty due to evolutionary and hereditary factors. Ancient anatomists have observed and recorded with descriptive precision asymmetrical manifestations in the human face. Moreover, sculptors and painters of the different epochs (Hellinistic, Roman epoch and Renaissance) not only have known the display of asymmetry in the human body but have also reproduced it in their works of art. For example, in the head of the famous Venus of Milo the left eyelid slit, the left ear are placed higher and the left facial half is more narrow than the right one [2]. In this case the realistic reproduction of the asymmetry in the face is the "minute" and hard-to-perceive detail that give life to the work.

The aim of the present study is to make an attempt for quantitative characterization of the degree and location of facial asymmetry in the face of the modern Bulgarian. This also stems from the fact that metric data about the asymmetry of the human face are very scanty [5, 7, 8].

The idea for quantitative determination of the degree of asymmetry in the human skull belongs to the late Prof. Kadanov, co-author of many data published earlier [3, 4]. This underlay the grounds for the study of the manifestation and degree of asymmetry in the live human face for the needs of the plastic anthropological reconstruction of the head soft tissues after the skull [9, 10].

Materials and methods

The investigations were carried out on 130 grown-up individuals — students at the Medical Academy — Sofia aged 20—22 years, 63 women and 67 men. 28 facial dimensions were measured in the investigated individuals (Fig. 1) — 20 out of them — bilaterally: 7 — horizontal, 6 — vertical and 7 — oblique ones. The better part of the dimensions were introduced by us using the classical anthropometric points [1, 6, 10]. A cephalometric set was used.

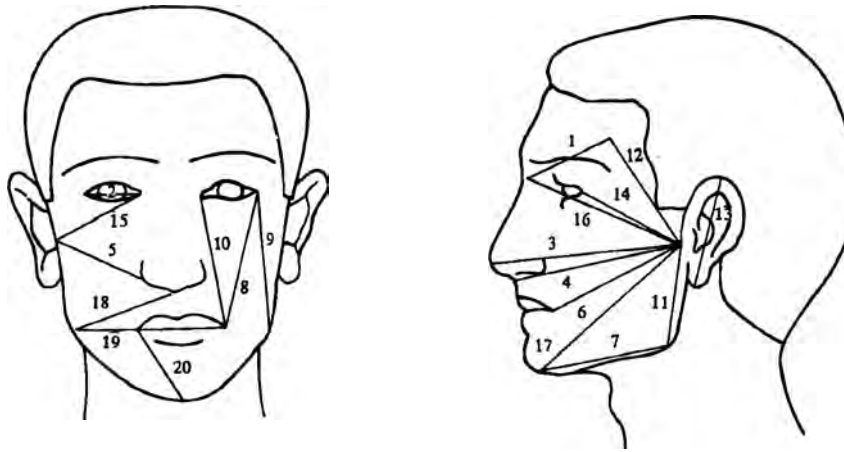

Fig. 1. Anthropometrical points and dimensions

The data relating to the degree of asymmetry were biostatistically processed after the method of Kadanov — Yordanov: full symmetry (0 mm difference), aproximate symmetry (difference ± 1 mm), overall symmetry (+1,0, —1) and asymmetry (difference 2, 3, 4 and more mm). The asymmetry with prevalence to the right is designated with "+" and the one to the left with "—". The values of the absolute differences and the per cent deviation between the values to the left and the ones to the right have been calculated (Table 1 and 2).

T a b l e 1. Per cent distribution of the differences in mm between the facial dimensions, n=63, ♀

Dimensions	0	+1 mm	—1mm	—1, +1 mm	—1, 0 +1 mm	2, 3, 4 ... mm
Horizontal						
1. Frontotemporale — Nasion	11,11	9,52	7,94	17,46	28,57	71,43
2. Entokanthion — Ektokanthion	65,08	11,11	17,46	28,57	93,65	6,35
3. Tragion — Pronasale	9,53	15,87	11,11	26,98	36,51	63,49
4. Tragion — Subnasale	14,29	14,29	6,35	20,63	34,92	65,08
5. Tragion — Alare	12,70	7,94	4,76	12,70	25,40	74,70
6. Tragion — Cheilion	11,11	6,35	11,11	17,46	28,57	71,43
7. Gonion — Gnathion	6,35	9,52	9,52	19,05	25,40	74,60
Vertical						
8. Ektokanthion — Cheilion	12,70	7,94	11,11	19,05	31,75	68,25
9. Ektokanthion — Gonion	12,70	9,52	6,35	15,87	28,57	71,43
10. Entokanthion — Cheilion	22,22	7,94	7,94	15,87	38,10	61,90
11. Tragion — Gonion	6,35	7,94	14,29	22,22	28,57	71,43
12. Tragion — Frontotemporale	11,11	12,70	7,94	20,63	31,75	68,25
13. Superaurale — Subaurale	22,22	4,76	19,05	23,81	46,03	53,97

Oblique						
14. Tragion — Ektokanthion	7,94	19,05	23,81	42,86	50,79	49,21
15. Tragion — Entokanthion	19,05	9,52	19,05	28,57	47,62	52,38
16. Tragion — Nasion	15,87	20,63	15,87	36,51	52,38	43,62
17. Tragion — Gnathion	7,94	9,52	6,35	15,87	23,81	76,19
18. Gonion — Subnasale	20,63	11,11	14,29	25,40	46,03	53,97
19. Gonion — Cheilion	11,11	14,29	11,11	25,40	36,51	63,49
20. Gnathion — Cheilion	17,46	14,29	22,22	36,51	53,97	46,03

T a b l e 2. Per cent distribution of the differences in mm between the facial dimensions, n=67, ♂

Dimensions	0	+1mm	—1 mm	—1, +1 mm	—1, 0, +1 mm	2, 3, 4... mm
Horizontal						
1. Frontotemporale — Nasion	25,34	19,40	19,40	38,81	64,18	35,82
2. Entokanthion — Ektokanthion	58,21	19,40	10,45	29,85	88,06	11,94
3. Tragion — Pronasale	16,42	8,96	7,46	16,42	32,84	67.16
4. Tragion — Subnasale	7,46	7,46	13,43	20,90	28,36	71,64
5. Tragion — Alare	20,90	8,96	16,42	25,37	46,27	53,73
6. Tragion — Cheilion	23,88	10,45	10,45	20,90	44,78	55,22
7. Gonion — Gnathion	17,91	7,46	14,93	22,39	40,30	59,70
Vertical						
8. Ektokanthion — Cheilion	17,91	16,42	4,48	20,90	38,81	61,19
9. Ektokanthion — Gonion	10,45	19,40	8,96	28,36	38,81	61,19
10. Entokanthion — Cheilion	26,87	13,43	10,45	23,88	50,75	49,25
11. Tragion —Gonion	8,96	7,46	19,40	26,87	35,82	49,25
12. Tragion — Frontotemporale	14,93	8,96	14,93	23,88	38,81	61,19
13. Superaurale — Subaurale	31,34	16,42	17,91	34,33	65,67	34,33
Oblique						
14. Tragion — Ektokanthion	7,46	7,46	8,96	16,42	23,88	76,12
15. Tragion — Entokanthion	16,42	8,96	17,91	26,87	43,28	56,72
16. Tragion — Nasion	16,42	11,94	14,93	26,87	43,28	56,72
17. Tragion —Gnathion	14,93	13,43	16,42	29,85	44,78	55,22
18. Gonion — Subnasale	8,96	17,91	16,42	34,33	43,28	56,72
19. Gonion — Cheilion	7,46	7,46	22,39	29,85	37,31	62,69
20. Gnathion — Cheilion	20,90	11,94	14,93	26,87	47,76	52,24

Results and discussion

The horizontal dimensions in women show the highest degree of symmetry 93,65 per cent in the width of the eyelid slit and lowest 25,40 per cent for the distance from tragion to alare and gonion-gnathion. In four of dimensions (with a difference of 1 mm), the asymmetry is prevalent to the right, in two — it is to the left and in 1 there is total identity. According to the vertical dimensions the auricle is most symmetrical (46,03 per cent) and the lowest degree of symmetry is observed in the Ektokanthion — Gonion and Tragion — Gonion (28,57 per cent)distances. In three of the dimensions (differing 1 mm) the asymmetry is prevailing to the left, in two — it is to the right and in 1 identity is observed.

According to the oblique dimensions the highest symmetry is recorded in Gnathion —Cheilion (53—97 per cent) with close values for Tragion — Nasion and Tragion — Ektokanthion.

In four of the dimensions the differences of 1 mm are in the trend to the left and in three — to the right.

The tendencies for asymmetrical manifestations in the males are similar to the ones in the females although in another per cent distribution.

The comparison of the per cent distribution of the differences between men and women shows that in four of the horizontal dimensions the symmetry in the representatives of the male sex is greater. Males are more symmetrical in all six vertical dimensions. In the oblique dimensions however, five out of the seven are marked by more symmetrical in the female sex.

The per cent distribution of the absolute differences shows that in the different dimensions full symmetry (difference = 0) is recorded in 6,4 per cent to 65 per cent of the cases. In fourteen dimensions the per cent of full symmetry is higher in men and in other seven — it is higher in women. The per cent distribution of the values shows a statistically significant symmetry (0 ± 1 mm) to the some degree and in the same dimensions as in the absolute symmetry. In women the vertical dimensions are most symmetrical followed by the oblique and horizontal ones. In the males the symmetry in the horizontal dimensions is better pronounced followed by the ones of the vertical and oblique dimensions. A genuine asymmetry (differences of 2 and more mm) can be found more frequently in women (in 14 of the dimensions)than in males (in 7 of the dimensions).

Conclusion

The summarized survey of the data for the genuine asymmetry (differences of 2, 3, 4 and more mm) direct our attention to the lower segment of the lateral part of the face — the space between Tragion—Gonion—Gnathion and to the one of Ektokanthion — Gonion — Cheilion. The findings of the higher localization of the left auricle and left eyelid slit are in full accord with the established higher localization of the left meatus acusticus externus and left orbit in precious craniologocal studies of ours [4]. Moreover the identity of the dimension Tragion — Gonion on the right and on the left and of Gonion — Gnathion is the lowest of all studied dimensions in women and Tragion — Subnasale, Tragion — Ektokanthion and Gonion — Cheilion is lowest in men.

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An attempt for anthropometric assessment of morpho-functional adaptation specific for different labour physical activities

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The aim of the paper is to assess the metrical changes in basic morpho-functional anthropological limbs' features, depending on the specificity of the physical activity in three professions in which the movements, the loading and the static efforts are combined in different manner. Nine representative male groups, each consisting of 100 persons, defined by profession and length of service are investigated. Data about 5 standard circumferences and 5 features which characterized the muscles and the muscles-fat ratio are studied. The changes in the investigated features are evaluated as according to the specificity of labour physical activity, and so according to the length of service — respectively up to 10, 20 and over 20 years. The results show that in work connected more with motor activity dominates the muscle component and in work connected mainly with loading and static efforts dominates the fat component in the muscle-fat ratio.

Key words: anthropometry, morpho-functional adaptation, different labour physical activi limbs' features.

Introduction

The limbs' circumferences are among the most ecosensitive features in man's anthropometric characteristics. The main factors, which contribute to metrical changes in the postnatal onthogenesis of these features are age and specificity of the physical activity in life, labour and sports [1, 2, 4, 5]. While age changes are comparatively well studied [3, 6], data on morpho-functional adaptation which is influenced by different physical activity are scarce.

The aim of this study is to assess the metrical changes in basic morpho-functional anthropological limbs' features, depending on specificity and duration of impact in three professions in which the motive activity, loading and static efforts are combined in different manners.

Materials and methods

A part of the data from a general anthropological investigation of representative groups of workers with different professions, distinguished by the specificity of the labour physical activity, is used for analysis and assessment in this paper. The analyzed data cover 900 males grouped in 9 age-professional groups, each 100 persons, as follows: clerks (financial-accounting personnel — C), millwrights (fitters and mechanics — M) and builders (brick-layers and plastereres — B). The professions are chosen so that each of them has specific physical activity in the work process. Three age groups of each profession are investigated: I (20—30 years), II (31—40 years), and III (41—55 years). These age groups give information also about the length of service in the corresponding profession, as by stipulation all the tested workers have not changed it. That is how the representatives from the first age group have 10 years of experience, from the second age group — 20 years of experience and from the third age group — more than 20 years of experience. Each person's data about 10 features are investigated: Arm circumference — relaxed, Arm circumference — contracted, Contractive difference, Muscle circumference of arm (MCA), Muscle-fat ratio of arm (MFRA), Forearm circumference, Muscle circumference forearm (MCF), Muscle-fat ratio of forearm (MFRF), Thigh circumference, Leg circumference. The muscle circumferences and the Muscle-fat ratios are calculated by formulas, used in the sports anthropology [7], and the results from their study are new in the general physical anthropology. In their calculations, data about the corresponding standard skin folds (SF) are used. The SF values are taken from the general investigation, but as they are not analyzed in this paper, their metrical values are not included in the Table 1. These are the formulae:

$$\mathrm{MCA} = \pi\left(\frac{\text{Arm circumference}}{\pi} - \frac{\mathrm{SF}_{\text{triceps}} + \mathrm{SF}_{\text{biceps}}}{2}\right),$$

$$\mathrm{MFRA} = \mathrm{MCA}\Big/\left(\frac{\mathrm{SF}_{\text{triceps}} + \mathrm{SF}_{\text{biceps}}}{2}\right),$$

$$\mathrm{MCF} = \pi\left(\frac{\text{Forearm circumference}}{\pi} - \mathrm{SF}_{\text{forearm}}\right),$$

$$\mathrm{MFRF} = \mathrm{MCF}/\mathrm{SF}_{\text{forearm}}.$$

The data for the studied features are processed variational-statistically and the measurement unit for circumferences is cm, and for the Muscle-fat ratio — per cent.

Analysis, discussion and results

To give an account of and to evaluate the presence or absence of morho-functional adaptation, depending on the specificity of labour physical activity, we analyzed comparatively the metrical differences in the particular features in interage as well as in interprofessional aspect. The graphics representing age curves for the particular features in the three professional groups give clear idea about the biological meaning

T a b l e 1. Metrical data

Features	Professional groups	21—30 years				31—40 years				41—55 years			
		$\bar{x}$	σ	m	v	$\bar{x}$	σ	m	v	$\bar{x}$	σ	m	v
Arm circumference — relaxed	C	28,92	2,37	0,23	8,19	30,50	2,81	0,27	9,19	30,83	2,52	0,24	8,17
	M	29,18	2,42	0,24	8,29	31,31	2,32	0,22	7,40	30,22	2,51	0,24	8,30
	B	29,53	2,89	0,28	9,78	30,09	2,90	0,28	9,63	30,60	2,74	0,26	8,95
Arm circumference — contracted	C	32,06	2,64	0,26	8,23	33,71	2,97	0,28	8,81	33,80	2,74	0,27	8,10
	M	32,42	2,59	0,25	7,98	34,37	2,51	0,24	7,30	33,12	2,69	0,26	8,12
	B	32,84	3,22	0,31	9,80	33,02	3,10	0,30	9,38	33,79	2,90	0,28	8,58
Contractive difference	C	3,14	0,99	0,10	31,52	3,15	0,91	0,09	28,88	2,97	0,89	0,09	29,96
	M	3,23	0,92	0,09	28,48	3,06	0,94	0,09	30,71	2,90	0,84	0,08	28,96
	B	3,31	0,98	0,09	29,60	2,93	0,80	0,08	27,30	3,19	0,97	0,09	30,40
Muscle circumference of arm	C	28,25	2,28	0,23	8,07	30,41	2,47	0,25	8,12	30,26	2,48	0,24	8,20
	M	28,54	2,24	0,22	7,85	30,68	2,22	0,22	7,24	29,47	2,19	0,22	7,43
	B	28,90	2,69	0,26	9,31	30,02	2,48	0,25	8,26	29.73	2,81	0,28	9,72
Muscle-fat ratio of arm	C	10,93	4,02	0.41	36,78	15,52	4,39	0,45	28,29	10,34	3,65	0.36	35,30
	M	13,66	5,08	0,51	37,19	13,90	3,99	0,40	28,70	10,64	3,73	0,37	35,06
	B	11,71	4,66	0,46	39,80	16,18	4,56	0,46	28,18	10,21	4,27	0,41	41,82
Forearm circumference	C	26,59	1,57	0,15	5,90	28,00	1,87	0,18	6,67	28,04	1,71	0,17	6,09
	M	27,38	1,64	0,16	5,98	28,83	1.78	0,17	6,17	27,92	1,60	0.16	5,73
	B	27,59	2,03	0,20	7,35	28,16	1.98	0,19	7,03	28,28	1,82	0,17	6,43
Muscle circumference of forearm	C	24,87	1,44	0,14	5,79	26,44	1,73	0,17	6,54	26,02	1,42	1,38	4,57
	M	25,86	1,39	0,14	5,38	27,14	1,63	0,16	6,00	25,92	1,50	0,15	5,79
	B	25,77	1,68	0,16	6,52	26,76	1,78	0,17	6,65	26,26	1,52	0,15	5,79
Muscle-fat ratio of forearm	C	16,22	5,46	0,54	33,66	18,47	5,56	0,54	30,10	14,32	4,68	0,45	32,68
	M	19,14	6,24	0,61	32,60	17,27	4,57	0,44	26,46	15,73	6,68	0,65	42,47
	B	16,18	5,72	0,56	35,35	19,56	5,00	0,49	25,56	16,16	6,12	0,50	37,87
Thigh circumference	C	53,37	4,01	0,39	7,51	55,14	4,13	0,39	7,49	54,28	3,82	0,37	7,03
	M	52,34	4,51	0,44	8,61	54,80	4,73	0,46	8,63	52,83	3,96	0,39	7,49
	B	53,29	4,42	0,43	8,29	51,93	4,49	0,43	8,64	53.73	4,02	0,39	7,48
Leg circumference	C	36.80	2,60	0,25	7,06	37,61	2,87	0,27	7,63	37,63	2,48	0,24	6.59
	M	35,85	2,46	0,24	6,86	37.24	2,66	0,26	7.14	36,52	2,63	0,26	7,20
	B	35,60	2,60	0,25	7,30	36,24	3.28	0,32	9,05	36,67	2,79	0.27	7,60

of the found intergroup differences (Fig. 1). In interage aspect the three professional groups show that both arm circumferences, relaxed and contracted, have lowest values in the first age group, and in the second age group they increase remarkably. In the third age group the increase continues in a smaller degree for millwrights and clerks, while for the builders Arm circumferences sharply decrease. The other three circumference of the upper limb — Muscle circumference of arm, Forearm circumference and Muscle circumference of forearm — show similar to the described age changes, but for them in the last age group a general trend to decrease is present. In medical-biological aspect this decrease of the circumferences of the upper limb in the third age group reflects age conditioned changes much more than changes caused by professional impact. After analyzing the data for the five Circumference features of the upper limb in interprofessional aspect, again most interesting biological information is given from the metrical interprofessional differences in the second age group. Millwrights are with highest values for the three Arm Circumferences, builders are with lowest ones, and the clerks take medial position. But the clerks still have lowest values for the Forearm circumference in the first and third age groups, the millwrights keep the leading position and the builders are in the middle. Obviously the Arm and the Forearm participate in different ways in the realization of the three types of labour physical activity, and they show differentiated interprofessional distinctions. On the other hand, the similarity of the interage and interprofessional differences among the values for Muscle circumferences and these for relevant segments of the upper limb general Circumferences, shows that these features give biological information, wich is relatively more dependent on the wholebody measures.

The Leg and Thigh circumferences' values show specific type of interprofessional differences. They are highest for the clerks, lowest for the builders and middle for the millwrights. Hypodynamics in office work, whis is accompanied with many static efforts, has definitively contributed to bigger circumferences of the clerk's lower limbs. For the builders, whose work is accompanied with big and various motive activity of the lower limbs, a trend to optimum reduction of their volume is determined. The millwrights most often work standing and their lower limbs take up indirectly the weights accompanying their work. The heavy static efforts needed to keep the standing work pose, additionally complicated with the weights, has probably contributed to the lower limbs' mass increase.

The results, at present available, generally describe the upper and lower limbs' volume and definitively give information about professionally dependent metrical changes. But using them it is not possible to specify which of the components of the circumference features contribute to the difference found. Such a possibility occurs after the Muscle-fat ratio of arm and forearm data analysis. In interage and in interprofessional aspect these data show specific intergroup differences and enriches the information about the upper limbs' morpho-functional adaptational possibilities. According to the MFR calculating formulae — MC/SF — the higher index values refer to muscle component superiority over the fat component in the ratio. The age curves for both Muscle-fat ratios show lowest values in the last, third, age group in all three professional groups, which means that during the period 40—55 years the subcutaneous fat tissue's relative share is higher than the corresponding muscles of the upper limbs. This muscles have their most vigorous growth among 30 and 40 years, when both work and life physical loading are biggest and in general stimulate superiority of the muscle component over the fat one. The only exception is the Muscle-fat ratio of Forearm for the millwrights, where it dominates since first age group. Interprofessional differences of these features are also mostly pointed in the second age group. But during this period every feature shows specific, according to

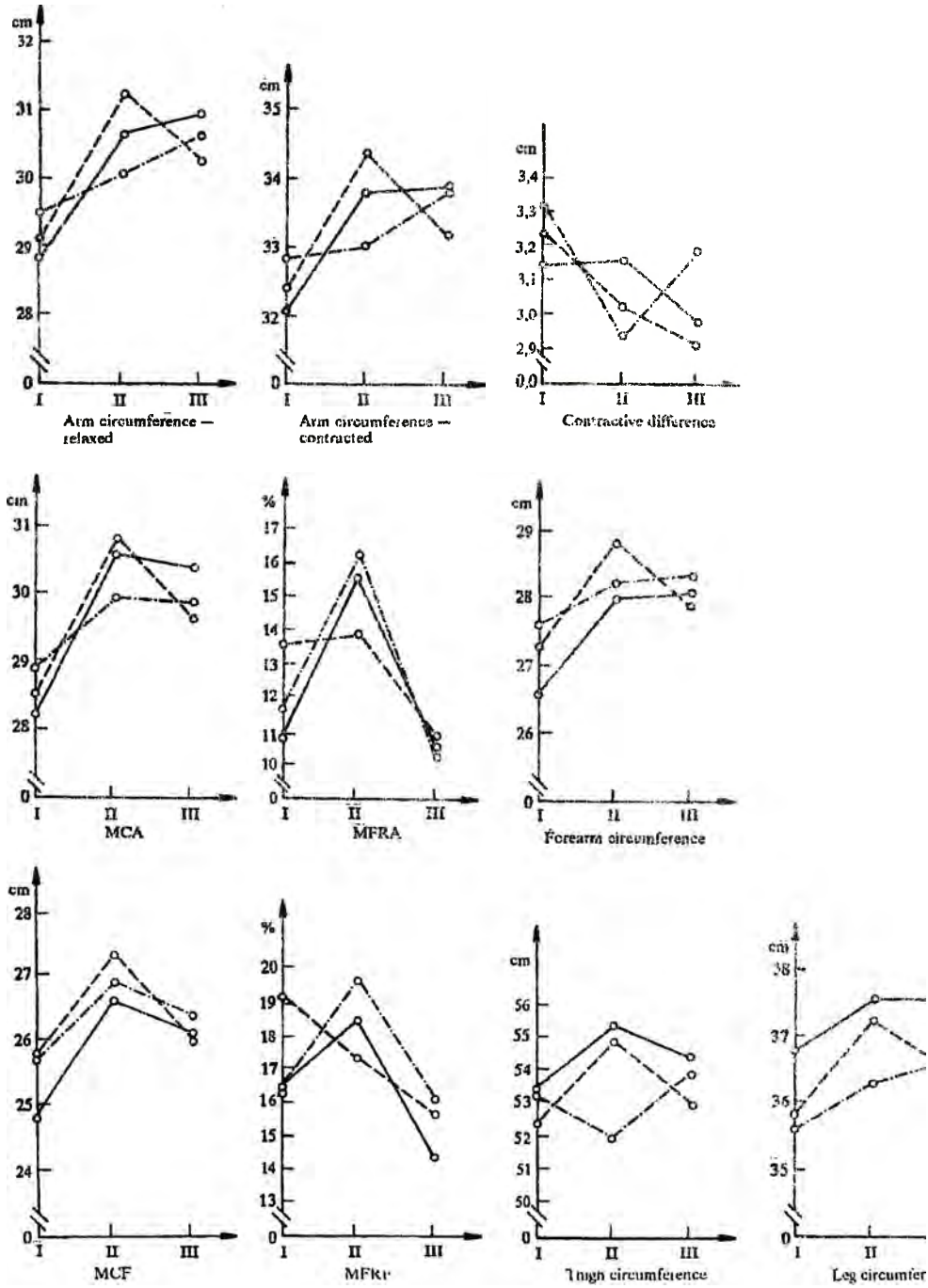

Fig. 1. Metrical differences (about 10 features) according to age and profession
Profession groups: — C; — — —M; —.—.—B; Age groups: I — 20÷30; II — 31÷40; III — 41÷55

us, professionally conditioned ratio of muscle and fat component of the Arm and Forearm circumferences. Millwrights, who are representatives of the physical labour, consisting of dominating static efforts for lifting and holding up heavy weights and comparatively little motive activity, have biggest part of the fat component in the Muscle-fat ratio in intergroup aspect. Obviously their fat tissue is necessary as a specific energy source for the work of mostly weighty and statically loaded up relevant muscles. For the builders the circumferences of the upper limb have least subcutaneous fat tissue, which reflects their labour physical activity, mainly connected with motive operations of higher volume, and the weights are smaller than these for the millwrights. For the clerks the Muscle-fat ratio has medial values, i. e. the participation of both circumference components is relatively equivalent. This ratio seems to be optimal for performing physical labour, mainly connected with static efforts, which must provide the precise manipulations in manuscript, typescript and calculation.

The contractive difference of the arm has independent type of interage and interprofessional metrical distincts. Though at first sight it may seem illogical, in the second age group, man's most active labour period, the clerks have biggest contractive difference and the builders — smallest. From other representative and extended comparative examinations we received similar data, showing the non-conformity to the name of the feature and the biological information which it gives. That gives us the reason to suggest a new meaning to this feature's name. The received information about the contractive difference, compared with the one about the Muscle-fat ratio of arm shows that it does not describe the functional possibilities of the armpit muscle independently, but the possibilities of all arm's soft tissues skin, hypodermic tissue, muscles, blood-vessels to strain and increase their mass as much as possible.

Summarized the results in this study show that the limbs' circumference features and the proportions between the muscles and the subcutaneous fat tissue definitely have the abilities for specific adaptational changes under the various labour physical activities influence. Muscle-fat ratio of arm and forearm are with highest ecosensibility, and in labour activity, connected more with motive operations the muscle component dominates in these proportions, while in labour activity, connected mainly with static efforts and weight loading, the fat component dominates.

In medical-biological aspect these results show that the purposive anthropologic investigations can be useful in evaluating the adaptation-compensatory changes in the locomotory system under the circumstance of different labour physical activity.

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Plantar dermatoglyphics in healthy children

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Dermatoglyphic investigation of soles of 806 clinically healthy Bulgarian children and adolescents (406 boys and 400 girls) was carried out. The frequencies and the type of pattern on separate areas of soles (after C u m m i n s, M i d l o, 1961) as well as the longitudinal creases (after M u t a f o v, T o r n j o v a, 1977) were analysed. The data were separately for both sexes and for both soles. Bilateral and intersexual differences about the frequency of pattern types were established on some plantar areas. The results were important for population genetics, on one hand, and, on the other hand, for the clinical practice as a basis for comparison and interpretation of the results from different inborn and inherited diseases.

Key words: plantar dermatoglyphics, healthy individuals, intersexual and bilateral differences.

The plantar dermatoglyphics is less studied in comparison to the dermatoglyphics of hands both in norm and in pathology. On the one hand, it is due to the difficulties with the procedure of print-taking and, on the other hand, the fact that there are not so many variations in the dermatoglyphic features. There are few publications on plantar dermatoglyphic characteristics in studies of separate populations [3, 7, 8, 12], of paternity [9] and of some chromosomal diseases [2, 4, 10]. Some of our publications on plantar dermatoglyphics in some groups of defective children are the only ones in Bulgaria [4, 5].

The aim of the present study is to give a dermatoglyphic picture of the plantar surface of healthy Bulgarian individuals, as well as intersexual and bilateral differences.

The dermatoglyphic plantar prints of 806 clinically healthy childern and adolescents of both sexes (406 boys and 400 girls) were used as material for the present study. The frequency and the type of patterns on the separate areas of the plantar surface are analyzed. The reading of the dermatoglyphic prints is made after the method of C u m m i n s and M i d l o (1961) [1]. The sole areas are after Memorandum on Dermatoglyphic Nomenclature, 1968 [6] (Fig. 1). The intersexual and the bilateral differences are evaluated after the Student's T-criterion.

Results

We analyzed the frequency and the type of patterns, of intersexual and bilateral differences separately for each area of the plantar surface.

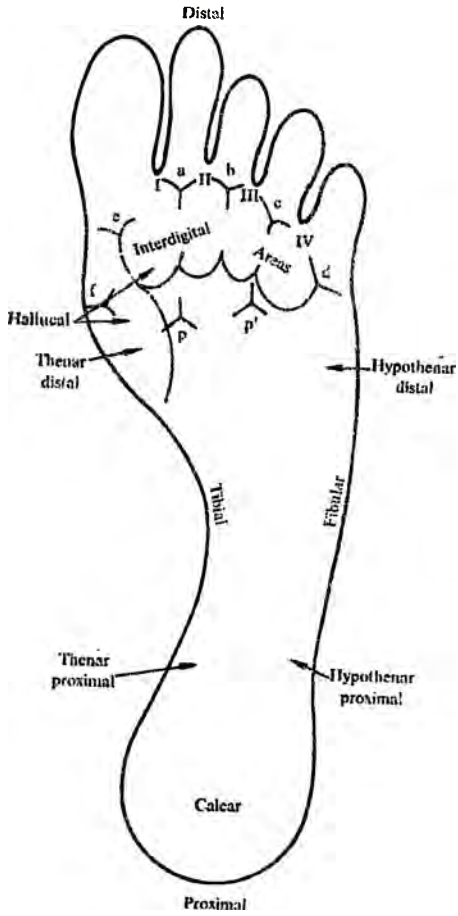

Fig. 1. Dermatoglyphic areas of the sole (by C u m m i n s, M i d l o, 1961)

Hallucal area (Table 1)

The most frequent pattern on the Hallucal area is the distal loop (L^d) on both soles in boys and girls. The second place is for the whorls (W) (in the sense of Galton), followed by the tibial loops (L^t). The other types of patterns are with relatively rare frequency. As for some patterns there are statistically significant intersexual differences. The distal loops are more frequent in girls on both soles than in boys, while the situation in the whorls is opposite, i. e. they are more frequent in boys ($p<0,05$). There are no statistically significant bilateral differences. However, distal loops in both sexes are with higher frequency on the right sole than on the left one, but the differences are statistically insignificant. There is a tendency showing that tibial loops on the left sole are with higher frequency than on the right one. The distribution of the three basic types of patterns (arches, loops, whorls) shows that the total frequency of the loops (L) is significantly higher in girls than in boys (♂ — 59,73 per cent, ♀ — 67,62 per cent), while the frequency of the whorls (W) is higher in boys (♂ —33,0 per cent, ♀ —24,62 per cent). The arches are with close frequencies in both sexes (♂ —6,53 per cent, ♀ —7,62 per cent). The total frequency of the true patterns (TP) on the Hallucal area (all types of loops, whorls and double patterns) is very high on both soles in boys and girls

T a b l e 1. Frequency (%) of patterns on the Hallucal area (Th^d/I_{IA})

Type of patterns	Boys			Girls		
	Right	Left	Both	Right	Left	Both
A^p	2,46	2,96	2,71	2,25	1,75	2,0
A^t	0,25	0,25	0,25	0,25	0,25	0,25
A^f	2,96	3,20	3,08	5,00	3,75	4,38
T	0,49	0,49	0,49	0,75	1,25	1,00
L^d	51,23*	46,55*	48,89*	61,00*	56,50*	58,75*
L^p	0,25	0	0,12	0	0	0
L^f	0,49	0,25	0,37	0,25	0,25	0,25
L^t	8,62	12,07	10,34	7,25	10,00	8,62
W	31,03*	31,52*	31,28*	21,75*	25,75*	23,75*
DP	1,72	1,72	1,72	1,50	0,25	0,88
V	0,25	0,74	0,50	0	0	0
O	0,25	0,25	0,25	0	0,25	0,12
TP	93,34	92,11	92,72	91,75	92,75	92,25

Note: DP — double patterns; TP — true patterns; * intersexual differences.

(from 91,75 per cent to 93,34 per cent). For these true patterns bilateral and intersexual differences are not established.

S u m m a r y. On the Hallucal area the loops (L) are 59,72 per cent in boys and 67,62 per cent in girls; the whorls (W+DP) are 33,00 per cent in boys and 24.63 per cent in girls; the arches (A+T) are 6,53 per cent in boys and 7,63 per cent in girls. The rest — 0,75 per cent in boys and 0,12 per cent in girls is formed by vestige (V) and open fields (0).

Second interdigital area (Table 2)

Most frequently there are no patterns in both sexes on the right and on the left soles in this area (♂ — 40,89 per cent, ♀— 44,43 per cent). If there are patterns they are from the type of proximal loop (L^p) (♂ —21,42 per cent, ♀ — 20,28 per cent). This proximal loops are more frequent on the left sole in boys, while in girls they are almost with equal frequency on both soles. The comparison between the right and the left soles regarding the tibial arches (A^t) indicates that they are more frequent on the right soles. The difference is significant in boys ($p<0,05$). There is a tendency showing that the fibular arches (A^f) are much more frequent on the left sole than on the right one. The distribution of the main types of papillar patterns (A, L, W) indicates that the whorls are with the lowest frequency (♂ — 5,79 per cent, ♀ — 6,38 per cent). The frequency of the arch patterns and the frequency of the loop patterns are close to one another (A ♂ — 27,22 per cent, ♀ —23,16 per cent; L ♂ — 25,98 per cent, ♀ — 25,40 per cent). There are no bilateral and intersexual differences. The frequency of the true patterns (L, W, DP) on this area is significantly lower than on the Hallucal area with an average of 60 per cent (♂ — 31,77 per cent, ♀ — 31,79 per cent). The true patterns are more frequent on the left sole in boys ($p<0,05$), while they are closer to one another in value on both soles in girls. There are no statistically significant intersexual differences in any pattern on this area.

S u m m a r y. On the second interdigital area the loops are 25,98 per cent in boys and 25,41 per cent in girls; the whorls (W+DP) are 5,79 per cent in boys and 6,38 per cent in girls; the arches (A+T) are 27,22 per cent in boys and 23,16 per cent in girls. The rest — 41,01 per cent in boys and 45,05 per cent in girls is formed by vestiges (V) and open fields (O).

T a b l e 2. Frequency (%) of the patterns on the II interdigital area

Type of patterns	Boys			Girls		
	Right	Left	Both	Right	Left	Both
A^p	0	0	0	0	0	0
A^t	15,52**	7,64**	11,58	11,25	7,52	9,39
A^f	13,79	17,49	15,64	12,00	15,29	13,65
T	0	0	0	0,25	0	0,12
L^d	3,94	5,17	4,56	6,50	3,76	5,13
L^p	19,21	23,64	21,42	21,00	19,55	20,28
L^f	0	0	0	0	0	0
L^t	0	0	0	0	0	0
W	2,96	3,94	3,45	4,00	5,01	4,50
DP	1,48	3,20	2,34	1,25	2,51	1,88
V	0	0,25	0,12	0,75	0,50	0,62
O	43,10	38,67	40,89	43,00	45,86	44,43
TP	27,59**	35,95**	31,77	32,75	30,83	31,79

Note: DP — double patterns; TP — true patterns; ** bilateral differences ($p<0,05$).

T a b l e 3. Frequency (%) of the patterns on the III interdigital area

Type of patterns	Boys			Girls		
	Right	Left	Both	Right	Left	Both
A^p	0	0	0	0	0	0
A^t	0	0	0	0	0	0
A^r	0	0	0	0	0	0
T	0	0	0	0	0	0
L^d	54,68*	49,75*	52,22*	47,75*	42,61*	45,18*
L^p	4,68	5,42	5,05*	7,25	8,02	7,64*
L^r	0	0	0	0	0	0
L^t	0	0	0	0	0	0
W	15,52	11,33	13,42	13,25	10,28	11,76
DP	0,49	1,23	0,86	0	0,75	0,38
V	2,22	1,97	2,10	0,75	1,00	0,87
O	22,41	30,30	26,35	31,00	37,34	34,17
TP	75,37**	67,73**	71,55*	68,25**	61,66**	64,96*

Note: DP — double patterns; TP — true patterns: * intersexual differences; ** bilateral differences.

Third interdigital area (Table 3)

The distal loop (L^d) is the most frequent pattern on both soles in boys and girls (♂ — 52,22 per cent, ♀ — 45,18 per cent). The second place is for the whorls (W+DP) but with significantly lower frequency (♂ — 14,28 per cent, ♀ — 12,14 per cent). The frequencies of the distal loops and of the whorls are higher on the right soles, than on the left ones in both sexes. It is worth noting that there are no cases with any arch pattern type. The total frequency of the true patterns is comparatively higher (♂ — 71,55 per cent, ♀ — 64,96 per cent). The true patterns are more frequent in boys than in girls, and more frequent on the right sole than on the left one ($p<0,05$). This is the only significant bilateral difference in both sexes.

S u m m a r y. On the third interdigital area the loops (L) are 57,27 per cent in boys and 52,82 per cent in girls; the whorls (W+DP) are 14,28 per cent in boys and 12,14 per cent in girls and the arches (A+T) are missing.

Fourth interdigital area (Table 4)

The absence of patterns is typical for this area (from 70,69 per cent to 83,22 per cent). If there is a pattern the most frequent one is the distal loop (L^d) which is with

T a b l e 4. Frequency (%) of the patterns on the IV interdigital area

Type of patterns	Boys			Girls		
	Right	Left	Both	Right	Left	Both
A^p	0,49	0	0,25	0	0,25	0,12
A^t	0,49	0,25	0,37	0,75	0,75	0,75
A^r	0	0	0	0	0	0
T	0	0	0	0	0	0
L^d	20,20*	18,47*	19,34*	13,50*	10,53*	12,01*
L^p	0,74	0,74	0,74	1,75	1,25	1,50
L^r	0	0	0	0	0	0
L^t	2,95	1,23	2,09	2,25	2,50	2,38
W	1,48	0,74	1,11	0,25	0,50	0,38
DP	0,25	0	0,12	0	0	0
V	2,71	1,97	2,34	1,25	1,00	1,13
O	70,69	76,60	73,64	80,25	83,22	81,73
TP	25,62*	21,18*	23,40*	17,75*	14,78*	16,27*

Note: DP — double patterns; TP — true patterns; * intersexual differences.

higher frequency in boys (♂ — 19,34 per cent, ♀ —12,01 per cent). This loop is a little bit more frequent on the right sole in both sexes. The frequency of the tibial loop (L^t) does not exceed 3 per cent while the other types of patterns are very rare, about and under 1 per cent. A double pattern (DP) is established on the right sole of one boy. The total frequency of the true patterns (TP) is relatively low (♂ — 23,40 per cent, ♀ —16,27 per cent). They are more frequent in boys and the difference is significant ($p<0,05$). The true patterns are more frequent on the right sole in both sexes.

S u m m a r y. On the fourth interdigital area the loops (L) are 22,17 per cent in boys and 15,89 per cent in girls; the whorls (W+DP) are 1,23 per cent in boys and 0,38 per cent in girls and the archess (A+T) are 0,62 per cent in boys and 0,87 per cent in girls.

Hypothenar (distal and proximal), thenar (proximal) and calcar area

Most often there is no pattern on the distal Hypothenar (Hy^d) on both soles in both sexes (♂ right — 68,47 per cent, left — 76,35 per cent; ♀ right — 71,50 per cent, left — 75,19 per cent). If there is a pattern the most frequent one is the tibial loop (L^t) (♂ right — 26,35 per cent, left — 21,18 per cent; ♀ right — 24,25 per cent, left —23,56 per cent). There are neither intersexual nor bilateral differences. A fibular loop (L^f) is established on the right sole of 3 boys and 1 girl while the same type of loop but on the left sole it found in only 1 boy. A whorl pattern (W) is established only on the

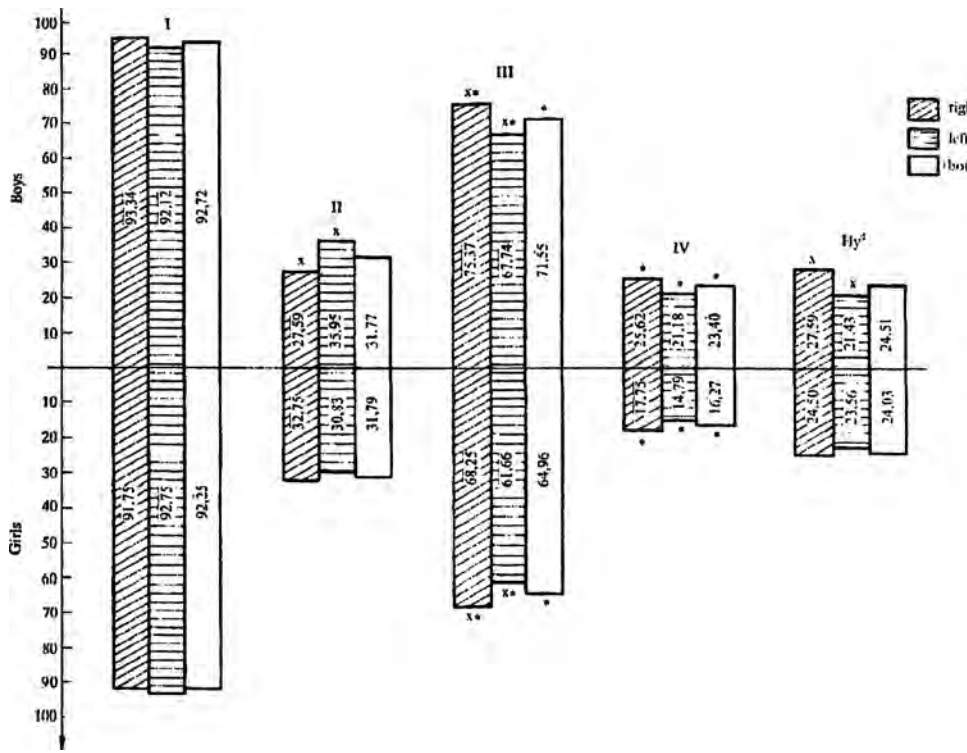

Fig. 2. The frequency of the true plantar patterns
x — bilateral differences; * — intersexual differences

Fig. 3. Plantar print of right sole

right sole of 1 boy. The frequency of the true patterns is equal in both sexes (♂ — 24,51 per cent, ♀ — 24,03 per cent).

There are practically no patterns on the proximal Hypothenar (Hy^p) (in more than — 99,00 per cent). Only two cases of tibial loop (L^t) on the right sole in boys are established while there is only vestige of pattern (V) in 1 girl.

Similar is the situation in the proximal Thenar (Th^p). There is no case with true pattern in both sexes. Vestiges of pattern (V) are established only in some children.

Most frequently there is no pattern on the Calcar area on both soles in both sexes. The true patterns are tibial loops (L^t) (♂ right — 1,72 per cent, left — 1,48 per cent; ♀ right — 1,0 per cent, left — 1,75 per cent) and a whorl on the right Calcar area of 1 boy. There are also some cases with vestiges of patterns (V).

Comparative analysis of the frequency of the true patterns on five of the plantar areas is carried out. Hallucal area, second interdigital area, third interdigital area, fourth interdigital area, Hypothenar distal are the areas with relatively more frequent true patterns. The formulae of the frequency patterns are equal in boys and girls on the right and on the left sole (Table 5). The most frequent true patterns are on the Hallucal area (♂ —92,72 per cent, ♀ —92,25 per cent), while they are most scarce on the fourth interdigital area (♂ — 23,40 per cent, ♀ —16,27 per cent) (Figs. 2, 3). Bilateral and intersexual differences are established on some of the plantar areas. For example, on the third interdigital area in both sexes and on the Hypothenar distal in boys only, there are more numerous patterns on the right soles than on the left one ($p<0,25$). On the contrary on the second interdigital area in boys the patterns frequency on the left sole is significantly higher than on the right one. Intersexual differences are related to the third and fourth interdigital areas where the frequency of the true patterns is higher in boys ($p<0,05$).

T a b l e 5. Formulae of the frequency of the true plantar patterns

Sole	Boys	Girls
Right	$Th/I>III>II>Hy^d>IV$	$Th/I>III>II>Hy^d>IV$
Left	$Th/I>III>II>Hy^d>IV$	$Th/I>III>II>Hy^d>IV$
Both	$Th/I>III>II>Hy^d>IV$	$Th/I>III>II>Hy^d>IV$

The frequency and the type of the longitudinal creases on soles are determined after the scheme of M u t a f o v and T o r n j o v a (1977) [4] (Fig. 4). It is established

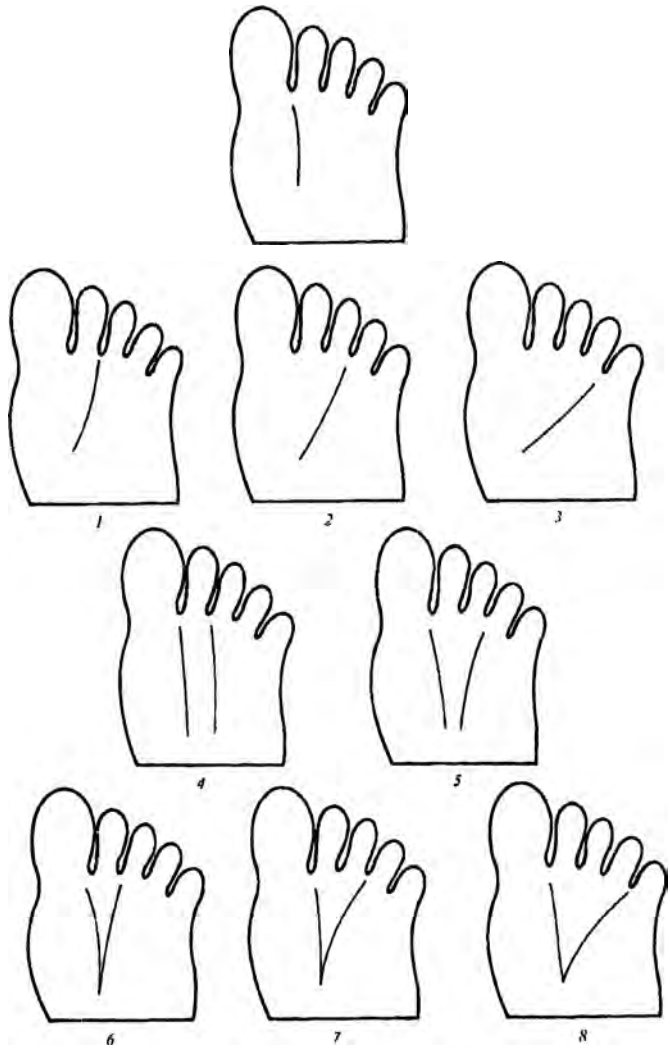

Fig. 4. Most frequent variations (*1–8*) of sole longitudinal creases (by M u t a f o v, T o r n j o v a, 1977)

that these creases are 26,72 per cent in boys and 15,64 per cent in girls. An intersexual difference is obvious ($p<0,05$). The distribution is nearly equal for both soles (♂ right — 26,35 per cent, left — 27,09 per cent; ♀ right — 15,75 per cent, left — 15,54 per cent). The classical form (on the top of the fig. 4) is with highest per cent in girls (44,75 per cent) while in boys No 1 is the most frequent one (51,59 per cent). The other types of longitudinal creases are with relatively low frequency.

As it is mentioned in the beginning of the paper there are no data about the plantar dermatoglyphics in Bulgaria and for this reason it is impossible for a comparison to be made. The comparison of our data is made with data from Polish [11], Slovak [8] and Hungarian [9] populations. In general, our results about the distribution of the frequency and the type of the patterns on the separate plantar areas are close to the above mentioned studies. The only difference is in the frequency

of the patterns on the Hypothenar distal in the work of P o s p i s i l [8] for Slovak population. Probably it is due to methodological differences.

S u m m a r y. The plantar dermatoglyphic status in particular and the general dermatoglyphic status including hands and soles of clinically healthy individuals is important for population genetics, on the one hand, and on the other hand, for the clinical practice as a basis for comparison and interpretation of the results from different inborn and inherited diseases.

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Hand clasping types of a population from North-West Bulgaria

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The types of hand clasping of 841 Bulgarians from two age groups (14—18 years old and 30—39 years old) have been investigated in three territorial regions of North-West Bulgaria — the Vidin, Montana (Mihailovgrad) and Vratza regions. It was established that the l-type is prevalent in the population under study. The observed differences between the R-type and L-type with regard to sex, age and territorial distribution in the three regions of North-West Bulgaria have proven to be statistically insignificant.

Key words: hand clasping, functional asymmetry, R-type, L-type.

One of the tests for determining the functional asymmetry of the hands as an expression of the functional asymmetry of the brain in the human is the so-called hand clasping e.g. the crossing of the fingers of the hands.

Lutz was the first [11] to describe two ways of crossing to the hand fingers in clasp. In the first case the right thumb is placed over the left one and is defined as the R-type in the other case it is the left thumb that is over and is defined as the L-type. Many authors have displayed an interest in that problem beginning from the dawn of the century till our day. The interest has been channelled mainly towards a clarification of the role of the factors determining the revealing of this trait and its relationship to other characteristics of the functional asymmetry of the brain. There is no inform opinion as to the nature of hand clasping though.

Like Lutz himself [11] many other authors later support the hypothesis of the genetic control over hand clasping [4, 5, 8, 12, 15]. Other authors claim that there is not good evidence for genetic control [3, 14]. L a i and W a l s h [9] assume it as a mere habit rather than a thing genetically pre-determined. L e g u e b e [10] envisages a combined influence of the factors in the a assessment of the hand clasping types. P o n s [12], F r e i r e - M a i a and A l m e i d a [6] note a dependence between age and this feature. B e c k m a n and E l s t o n [1] do not find significant differences regard to sex as well as no record of regional variations. R h o a d s and D a m o n [13] do not establish any dependency on sex and age.

The start of studies of this kind was made B o e v and T o d o r o v [2] in our country who investigated 2638 individuals from three ethnic groups and find a much higher percentage of the R-type especially among Bulgarians compared to other

investigations. M u t a f o v [16] has explored a control group of 1500 healthy children where the L-type is predominant. Later K a r e v [7] studies 2100 students and also established a higher percentage for the L-type.

The comparatively small number of studies on hand clasping in our country and its unclarified character has set us on the path of carrying out this study. The purpose of the present study is to trace the frequency of the hand clasping types in the population from North-West Bulgaria depending on sex, age and residency.

Material and Methods

A total of 841 Bulgarians of both sexes have been investigated who inhabit and descend from three territorial regions of North-West Bulgaria: the Vidin, Montana (Mihailovgrad) and Vratza areas. The studied individuals were distributed into two age groups: 1) 14—18 years old — encompasses 89 boys and 156 girls, all students; 2) 30—39 years old — encompasses 301 males and 295 women.

The investigation was carried out after the conventional methods [10, 13]. The statistical processing includes the per cent distribution of the hand clasping types and the χ^2 method.

Results and Discussion

The L-type is prevalent both throughout the total population studied in North-West Bulgaria and among both sexes where the values are quite close (Table 1). The differences are statistically insignificant between the two types of hand clasping with regard to sex ($\chi^2=0{,}001 < \chi^2_{0{,}05(1)}=3{,}84$). The L-type is predominant in both sexes from the three regions its percentage being highest among the males from Montana and Vratza women (Table 2). The differences, however, are statistically insignificant in

T a b l e 1. Per cent distribution of the hand clasping types amid a population of North-West Bulgaria according to sex

Sex	R-type		L-type		N
	n	%	*n*	%	
Men	172	44,10	215	55,13	390
Women	200	44,35	251	55,65	451
Total	372	44,23	466	55,41	841

T a b l e 2. Per cent distribution of the hand clasping types amid a population of North-West Bulgaria according to residency

Region	Sex	R-type		L-type		N
		n	%	*n*	%	
Vidin	men	62	46,97	68	51,52	132
	women	53	44,54	66	55,46	119
Montana	men	57	41,30	80	57,97	138
	women	99	46,70	113	53,30	212
Vratza	men	53	44,17	67	55,83	120
	women	48	40,00	72	60,00	120

both sexes (for the males χ^2=1,01< $\chi^2_{0,05(2)}$= 5,99).

In the age group from 14 to 18 years of age the L-type is more frequent in both sexes the percentage being somewhat higher among the girls (Table 3). The difference between the R- and L-types with regard to the sex are statistically insignificant (χ^2=0,65< $\chi^2_{0,05(1)}$= 3,84). The L-type is also prevalent in the 30—39 years-of age group up and is higher in frequency among males (Table 3).The differences here are again statistically insignificant (χ^2=0,72< $\chi^2_{0,05(1)}$=3,84). 1% of the investigated males place the thumbs of both hand parallel to each other when crossing fingers. Upon comparing the two age groups it was established that the R-type per cent is increased in the second age group without gaining prevalence over the per cent of the L-type, however. These changes in the percentage of the R- and L-types are statistically insignificant in both sexes, especially in the male group (χ^2= 0,76< $\chi^2_{0,05(1)}$=3,84), for women χ^2=7,99< $\chi^2_{0,001(1)}$=10,83. The established age differences in the two types of hand clasping are possibly related to the changes in the way of living during the next phases of the ontogenetical development of man (the type of occupation, professional qualification,

T a b l e 3. Per cent distribution of the hand clasping types amid a population of North-West Bulgaria according to age group

Age group	Sex	R-type		L-type		N
		n	%	*n*	%	
14—18 years	men	36	40,45	53	59,55	89
	women	55	35,26	101	64,74	156
30—39 years	men	136	45,18	162	53,82	301
	women	145	49,15	150	50,85	295

T a b l e 4. Per cent distribution of the hand clasping types amid a population of North-West Bulgaria according to residency in the corresponding age group of 14—18 years of age

Region	Sex	R-type		L-type		N
		n	%	*n*	%	
Vidin	men	13	37,14	22	62,86	35
	women	16	36,36	28	63,64	44
Montana	men	22	42,31	30	57,69	52
	women	37	35,92	66	64,08	103
Vratza	men	1	50,00	1	50,00	2
	women	2	25,00	7	77,78	9

T a b l e 5. Per cent distribution of the hand clasping types amid a population of North-West Bulgaria according to residency in the corresponding age group of 30—39 years of age

Region	Sex	R-type		L-type		N
		n	%	*n*	%	
Vidin	men	49	50,52	46	47,42	97
	women	37	49,33	38	50,67	75
Montana	men	35	40,70	50	58,14	86
	women	62	56,88	47	43,12	109
Vratza	men	52	44,07	66	55,93	118
	women	46	41,44	65	58,56	111

etc). The L-type per cent is highest among the boys from the Vidin region and in the girls from the Vratza area (Table 4). The differences, however, are statistically insignificant in both sexes with respect to the territorial distribution (among boys $\chi^2=0,30 < \chi^2_{0,05(2)}= 5,99$ and for the girls $\chi^2=0,69< \chi^2_{0,05(2)}=5,99$). The R-type predominates in the males from the Vidin region and among the women from the Montana area (Table 5). The differences here are also statistically insignificant (for the males $\chi^2=2,14< \chi^2_{0,05(2)}=5,99$ and for the women $\chi^2=5,24< \chi^2_{0,05(2)}=5,99$). 2,06% from the males of the Vidin region and 1,16% from the Montana area place the thumbs of the two hands parallel to one another when crossing their fingers.

The data from the present study are quite close to the results from the investigations of Mutafov and Karev (Table 6).

T a b l e 6. Comparative data about the frequency of the hand clasping types

Population	Author	Sex	N	Hand clasping (%)	
				R-type	L-type
Bulgarians(North	B o e v, T o d o r o v	men	397	66,25	33,75
Bulgaria)	(1973)	women	354	59,71	40,39
Bulgarians	M u t a f o v	men	797	46,04	53,95
	(1981)	women	703	45,23	54,77
Bulgarians	K a r e v	men	1050	47,71	52,29
	(1993)	women	1050	46,29	53,71
Bulgarians (North-	Filcheva	men	390	44,10	55,13
West Bulgaria)	(present study)	women	451	44,35	55,65

The following conclusions can be drawn on the basis of the obtained results:

1) The frequency of the hand clasping types in the studied population corresponds to the parameters of the Europid race.

2) The biological factors sex and age exert an insignificant influence on the per cent distribution of the hand clasping types.

3) Residency, as a complex of geographical and socio-economical factors, which in our case coincides with the origins of both the mother and father sides of the investigated individuals is also of insignificant influence on the hand clasping types.

4) Categorical conclusions about factors underlying manifestation of hand clasping would be made possible in large scale familial and longitudinal studies yet to be performed.

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Seasonality of menarche in Bulgaria

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Data of monthly distribution of menarche in 799 school and university students (9 samples) are analysed and compared to data from 10 other samples. Two basic types of menarcheal seasonality in Bulgaria can be distinguished: 1) spring-summer accumulation plus a small winter peak — in more urbanized populations; 2) two periods of accumulation, summer and winter — in less urbanized populations. The results support the concept that influence of social environmental factors on menarche is stronger than the influence of natural environment.

Key words: menarche, seasonality of menarche, influence of social factors on menarche, urbanization and menarche.

Seasonal oscillations of biologic functions in man constitute a basic problem in human ecology. Here belongs also the phenomenon of seasonality of the first menstruation — menarche [7].

There are some data about seasonality of menarche in some maturation studies in Bulgaria [1, 4, 5, 6, 8]. But there is not a study dedicated especially to this problem.

The aim of the present study is to trace the total picture of seasonality of menarche in Bulgaria and its peculiarities in the different groups of the population.

Data of investigations in schoolgirls in Sofia (n = 241) and Smolyan (n = 110) and of university students in Sofia (n = 927), collected in 1984-1987 have been used. Students responces account for menarcheal distribution of about 9 years before the investigation (late 1970s). For analysis x^2-test has been used.

The question about the month of menarche was answered by 235 schoolgirls in Sofia (97,5 %), 84 — in Smolyan (76,4 %) and by 480 university students (51,8 %). These proportions are good for analysis [7]. It shows that seasonal irregularity of menarche in the samples and the differences between them and other studies data are statistically significant (Table 1).

Even at first glance can be detected differences in seasonality of menarche between girls from the so-called Sofia-A (daughters mostly of employees and intellectuals with university education and high living standard) and of Sofia-B (parents of lower educational and cultural level and lower living standard). In the first ones well expressed accumulation of menarcheal cases in winter-spring (December—March) is recorded and the summer maximum is absent (Fig. 1). In Sofia-B the accumulation is in summer (July-September) and in winter only a small peak can be observed (in January). Such a kind of distribution (accumulation in the hot season and a small

winter peak) is dominating in the analyzed samples and was called "type 1". A distribution of summer and winter accumulation is more rare and has been called "type 2". Such a distribution has been found in the schoolgirls from Smolyan — a December-January and July-September accumulation. The distribution of menarche in schoolgirls from Sofia-A has not been found in other samples and has been called "type 3".

All distributions in urban students are of the type 1. The difference in comparison to Sofia-B schoolgirls is that the accumulation begins earlier, in April or May and ends in August-September. So we named this type "1a". By the end of spring or the beginning of summer a decrease of menarcheal cases compared to the preceding month can be observed in all distributions. When they drop below the theoretically

T a b l e 1. Seasonality of menarche in the investigated girls (%)

Month	Theoretical level	Schoolgirls			Students (by residence)				
		Sofia-A	Sofia-B	Smolyan	Sofia (born in Sofia)	Sofia (migrants)	cities (over 25 000)	towns	villages
J	8,5	16,2	11,0	17,9	7,5	13,7	12,2	18,6	11,1
F	7,7	12,1	4,4	6,0	11,2	4,1	5,1	3,4	8,9
M	8,5	12,1	7,4	4,8	4,7	2,7	4,6	3,4	6,7
A	8,2	8,1	6,6	2,4	7,5	11,0	8,7	11,9	4,4
M	8,5	9,1	4,4	7,1	10,3	9,6	11,7	5,1	4,4
J	8,2	7,1	6,6	7,1	9,3	5,5	8,7	8,5	11,1
J	8,5	6,1	19,8	10,7	11,2	5,5	12,2	13,6	22,2
A	8,5	7,1	16,2	14,3	13,1	12,3	10,7	20,3	13,3
S	8,2	7,1	11,0	14,3	10,3	15,1	10,2	5,1	6,7
O	8,5	0,0	0,7	2,4	4,7	6,8	5,6	0,0	2,2
N	8,2	5,1	6,6	1,2	4,7	6,8	3,6	1,7	2,2
D	8,5	10,1	5,2	11,9	5,6	6,8	6,6	8,5	6,7
n		99	136	84	107	73	196	59	45
x^2		21,97	50,24	31,80	13,03	14,86	24,09	33,62	18,43
$p \leq$		0,01	0,001	0,001	0,1	0,1	0,01	0,001	0,05
Type		3	1	2	1a	1b	1a	1b	2

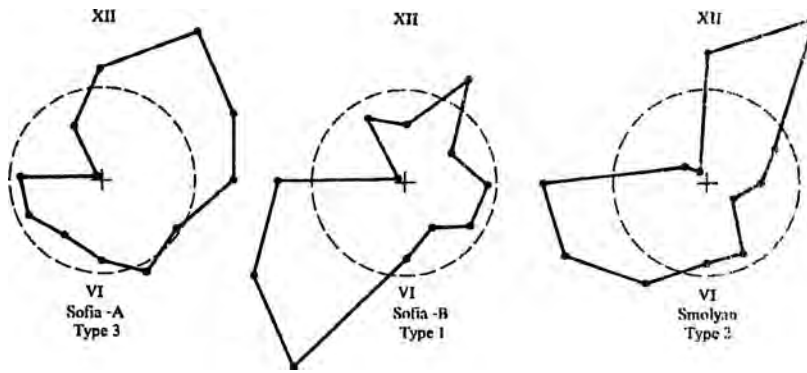

Fig. 1. Menarche in schoolgirls

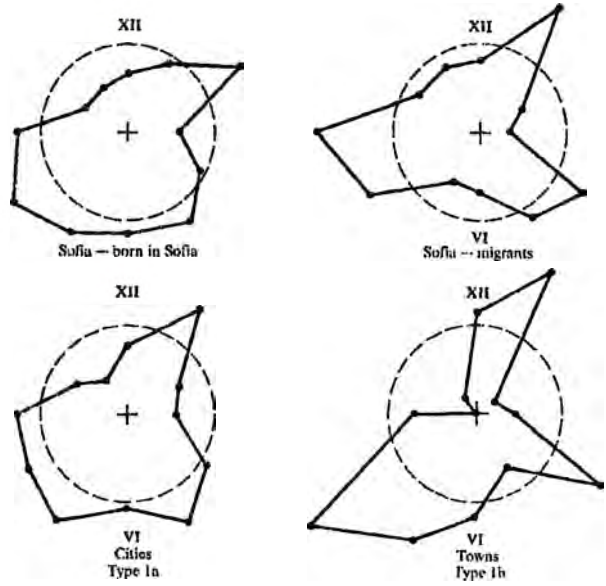

Fig. 2. Menarche in students in Sofia

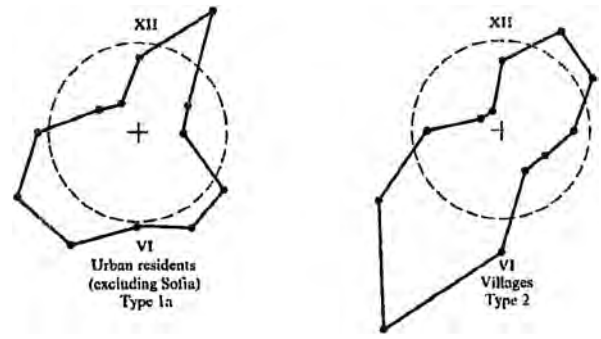

Fig. 3. Menarche in students outside Sofia

expected level the name "type 1b" has been introduced. It is typical of less urbanized samples (Fig. 2).

Village students present a distribution of type 2, with well expressed summer and winter accumulations (Fig. 3).

The review of former data reports that in Sofia the seasonality was of type 1 during the whole postwar period. In 1950s, however, it was of type 1b, as in the less urbanized samples later [1, 4, 6]. The towns of the district of Veliko Tarnovo and of Northeast Bulgaria in 1960s and 1970s show a seasonality of type 1b [2, 5]. The less urbanized populations in the same period (Haskovo, Haskovo villages, Blagoevgrad, Veliko Tarnovo villages) report distribution of type 2 or similar [4, 5, 8]. All this is in good concord with our study.

Closest to the dominating in our urban populations seasonality of type 1b is the one found in urban Georgian populations. An intermediate seasonality between type

1 and type 2 can be observed in Russian urban girls [7]. In Serbia, even in the city of Belgrade, the distribution is mostly of type 2 [3].

The specific type 3 in schoolgirls from Sofia-A (maturing most early of all analyzed samples) can be explained by the displacement of menarche to winter and spring in early maturing girls [7].

The results support the assumption about the role of the psychic factor in menarche [7]. The most common seasonality of type 1b can be explained by stresses and emotions in the beginning and the end of the holidays and classes. Autumn, the longest season without holidays, is always a period with minimum menarcheal frequency (in Bulgaria and abroad).

The results of this study show that in the urban population of Bulgaria since a long time the spring-summer accumulation of menarcheal cases plus a small winter peak is dominating (seasonality type 1). In the less urbanized populations two accumulation periods, summer and winter, can be observed (type 2). In early maturing girls of very good socio-familial conditions a winter-spring accumulation is found (type 3). The results support the conception for the prevalent role of social environment in the seasonality of menarche.

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Anthropological characteristics of a West Rhodope population based on dermatoglyphic data

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Dermatoglyphic studies were carried out in two regions situated in the central part of the West Rhodope Mountains — the West region (Dospat–Devin–Shiroka Luka) and the central one (Smolyan). A total of 570 males and 640 females was investigated. It was established that typologically according to the combinations of the features the Rhodope population under study can be qualified as Europeoid. According to the values of the dermatoglyphic features it belongs to the Southern Europeoids. The Southern Europeoid features are less pronounced in the West region and are considerably more strongly pronounced in the Central Rhodope region.

Key words: anthropology, ethnic dermatoglyphics, Southern Europeoid dermatoglyphic complex, Eastern dermatoglyphic complex.

There are no data about the Bulgarian population in the international handbooks on ethnic dermatoglyphics [7]. Partial studies using the specific methods of ethnic dermatoglyphics were carried out in the south-west part of the Central Rhodopes on the territory of the former Smolyan district. The results from these studies showed a slight shifting of the ordinary Europeoid type in eastern direction displayed by the variations of the eastern complex [3]. These data in the dermatoglyphic characteristics of the population from the mentioned region imposed an extension of the area of the dermatoglyphic investigation in the Rhodopes and the performance of a comparative typological analysis in order to trace the dispersal of the eastern complex.

Material and methods

In the present article are presented the results from a dermatoglyphic study of the population of two territorial groups situated in the central part of the West Rhodopes. One of the groups encompasses the population of the Dospat, Devin and Shiroka Luka region. Their binding into one group Dospat–Devin–Shiroka Luka group is justified by the fact that this population showed the same dermatoglyphic complex [2]. 400 males and 469 women belonging to this group were studied. The second

Fig. 1. Distribution of investigated individuals according to local origins

territorial group — the Smolyan one included the population from the Smolyan, Chepelare and Momchilovtsi region and was called for simplicity the central group. 170 men and 171 women of this group underwent the study (Fig. 1).

The dermatoglyphic imprints were processed after the method of C u m m i n s and M i d l o [1] and the proximal palmar triradii were determined after the scheme of A. S h a r m a [4]. The method of H e e t [6] was used in the data analysis. This method allows for obtaining various information out of the material related to: the typological assessment of the combinations of dermatoglyphic features represented by combination polygons; the determining of the total dermatoglyphic distance (DD) between the groups according to sums of features. This distance gives the possibility for establishing the differences between the groups; the determining of the Eastern complex (MC) and the introduction of the Southern complex (SC).

The 5 basic ethnic dermatoglyphic features were analyzed in the paper: viz. pattern intensity index (PII), main line, or Cummins, index (MLI), proximal palmar triradius (t), true hypothenar patterns (Hy), and accessory interdigital triradii (AIT).

Results and Discussion

The results obtained from the processing of the dermatoglyphic imprints are shown in Tables 1-6 and in Fig. 2 and 3. The analysis of the data showed that the population from both groups under study is characterized with an increased percentage of the non-delta pattern, with an increased number of circular patterns and a mean value of the PII in the scale of the Europeoid groups (Table 1). The Cummins index values are low in both groups this tendency being more persistent in the examined ones

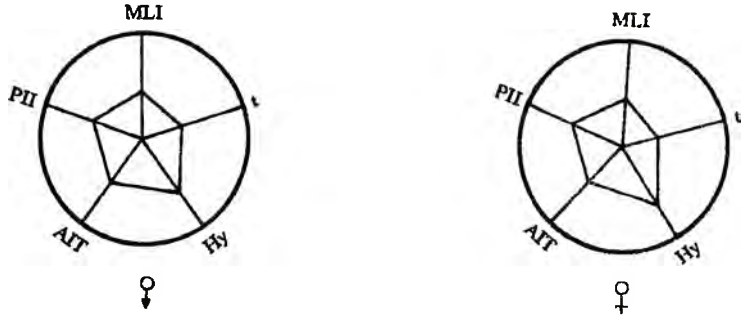

Fig. 2. Polygonic graphs showing the variation of dermatoglyphical features in the West Rhodope region

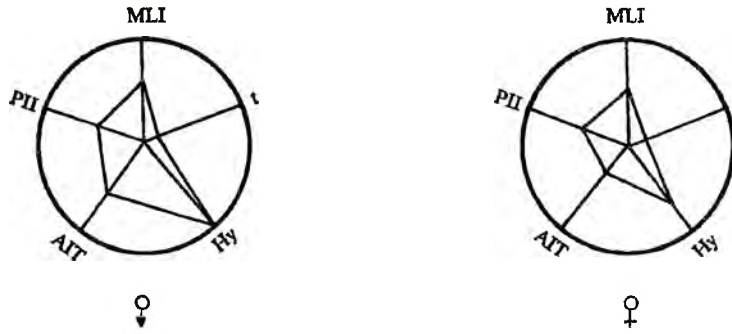

Fig. 3. Polygonic graphs showing the variation of dermatoglyphical features in the Central Rhodope region

from the West region (Tables 2-3). The percent content of the proximal palmar triradius among the population from the central region is considerably lower (45 %) than the one observed in the sample from the West Rhodope region (64 %) (Table 4). According to Heet's investigations, the value size of the proximal palmar triradius in the central group is closer to the minimum value for that feature in the Europeoid groups [6]. In spite of the differences according to this trait between the groups the per cent content of the proximal palmar triradius in the western territorial group is not high either and is within the normal scale for the Europeoid groups. The percentage of the true hypothenar patterns in both groups is high. It is conspicuous that this per cent is much higher in the central group (Table 5). In both Rhodope populations the expression of the Th_1 pattern is of average values (9-10 %). The pattern of the II interdigital pad is weakly pronounced (2-6 %). The frequency of the pattern of the IV interdigital pad is higher than the one of the III in both groups under study. The per cent of the accessory interdigital triradii in both Rhodope groups is of mean values but in the males of the Western group this feature is of lowered frequency (Table 5).

Summing up the data from the individual analysis we have to emphasize that according to the variations of the dermatoglyphic features the two territorial groups under study belong to the Europeoids and according to the values of these features they represent South Europeoids. These results were confirmed by the overall

T a b l e 1. Digital pattern (%) and pattern intensity index (PII) in Rhodope population

Group	Number and sex	A+T	R	U	R+U	W	PII
West Rhodope group	400 men	5,1	2,5	51,5	54,0	40,8	13,57
	469 women	6,3	2,0	55,1	57,0	36,7	13,04
Central Rhodope group	170 men	7,3	2,2	52,5	54,8	37,9	13,06
	171 women	7,0	2,3	56,3	58,6	34,2	12,71

T a b l e 2. Termination of the main line in a Rhodope population (%)

Line A

Group	Sex	1	2	3	4	5'	5''	6	7
West	men	2,1	0,4	46,9	19,9	29,4	1,3	—	—
	women	1,7	0,6	55,2	16,2	25,5	0,8	—	—
Central	men	1,7	0,8	48,5	13,8	31,9	2,5	—	0,7
	women	1,9	0,7	46,2	18,2	31,5	1,5	—	—

Line B

Group	Sex	3	4	5'	5''	6	7	8	9	X	O
West	men	0,7	0,9	29,4	26,5	1,0	40,0	0,3	1,0	0,1	0,1
	women	0,6	0,8	21,1	35,2	0,1	40,6	—	1,3	0,1	—
Central	men	—	0,3	15,8	37,4	—	45,0	0,3	1,2	—	—
	women	0,9	—	17,8	36,2	—	43,9	—	0,6	0,6	—

Line C

Group	Sex	5'	5''	6	7	8	9	10	11	X	O
West	men	1,1	12,9	0,1	23,4	0,2	35,6	0,2	1,2	19,6	5,5
	women	0,4	18,0	0,2	18,4	0,1	28,9	—	1,1	26,0	6,7
Central	men	—	12,9	—	22,3	—	37,1	—	1,2	23,8	2,6
	women	—	16,9	—	21,3	—	30,4	—	0,6	26,0	4,7

Line D

Group	Sex	7	8	9	10	11	13
West	men	6,2	0,2	37,5	3,0	53,1	—
	women	8,4	—	35,2	0,5	55,9	—
Central	men	5,5	—	33,0	0,9	60,0	0,4
	women	7,7	—	32,9	0,6	58,7	—

T a b l e 3. Types of the main' lines and Cummins index (MLI) in a Rhodope population

Group	Sex	Types of lines A				Types of lines D				MLI
		1	3	5	M_{A1-5}	7	9	11	M_{D7-5}	
West	men	8,0	71,1	20,9	3,26	14,3	45,4	40,3	9,52	7,99
	women	6,8	75,5	17,6	3,21	18,9	39,5	41,6	9,45	7,83
Central	men	7,3	68,5	23,8	3,32	12,9	39,7	47,3	9,68	8,19
	women	7,9	69,3	33,9	3,85	17,5	38,0	44,4	9,53	7,93

Table 4. Proximal palmar triradii in a Rhodope population (%)

Group	Sex	t	t'	t''	tt'	tt''	t't''	tt't''	o	tt	t't'	t''t''	tt''t''	t''t''	ttt'	ttt''
West	men	64,4	15,9	5,9	3,4	5,5	1,1	—	0,6	0,8	0,8	0,1	0,3	0,1	—	—
	women	55,3	23,0	5,6	4,6	5,3	1,6	0,2	1,1	1,4	0,6	0,1	0,1	0,1	0,3	0,3
Central	men	45,6	21,8	9,7	6,2	10,0	2,1	0,3	1,5	0,9	0,3	0,6	0,3	0,6	—	—
	women	45,3	23,4	10,8	6,4	8,2	1,5	0,3	0,6	1,2	0,9	0,3	0,3	0,3	0,6	0,3

Table 5. True palmar pattern and accessory interdigital triradii in a Rhodope population (%)

Group	Sex	Palmar pattern					Accessory interdigital triradii			
		Hy	Th_1	II	III	IV	II	III	IV	II–IV
West	men	31,5	9,4	5,9	21,4	41,5	5,6	1,0	17,5	24,1
	women	39,0	10,2	2,9	22,3	47,6	2,9	1,5	16,1	20,2
Central	men	46,2	9,1	5,9	25,9	47,9	5,9	2,9	20,6	29,7
	women	40,0	10,8	2,0	21,3	37,1	2,0	1,2	11,1	14,3

Table 6. Variations of the main dermatoglyphic features in a Rhodope population

Group	Number and sex	PII	MLI	t	Hy	AIT	Th_1	SK	MK
West	400 men	13,57	7,99	64,4	31,5	24,1	9,4	54,4	46,7
	469 women	13,04	7,83	55,3	39,0	20,2	10,2	53,9	46,6
Central	170 men	13,06	8,19	45,6	46,2	29,7	9,1	65,2	25.9
	171 women	12,71	7,93	45,3	40,0	14,3	10,8	54,8	43,8

comparison of the dermatoglyphic features in which the Southern and Eastern complexes were determined (Table 6). As an illustration to the overall comparison the combination polygons were used (Figs. 2 and 3). The analysis of the data from the overall comparison showed that according to the degree of expression of the South Europeoid traits the two Rhodope dermatoglyphic complexes are different. These differences are most strongly expressed in the males. Their total dermatoglyphic distance is rated into the category of "very large" (DD = 20,7). In women the total dermatoglyphic distance is rated into the category "small" (DD = 8,5). The analysis of the data was carried out separately for the men and for the women because of the presence of a significant sexual dimorphism in the Europeoids in the skin relief [5]. The higher values of the South Europeoid complex (65,2 %) and the much lower values of the eastern complex (25,9 %) in the men from the central group come as a result from the very low frequency of the proximal palmar triradius and the very high frequency of the true hypothenar patterns as well as because of the elevated values of the accessory interdigital triradii (Fig. 3). In the women from the same group this peculiarity is more clearly expressed with respect to the proximal palmar triradius. Most probably we are encountering a local combination of the features forming the South-Europeoid complex in the dermatoglyphic characteristics of the population from the central-Smolyan group* [2].

* I am expressing my deep gratitude to Prof. G. L. Heet from the Institute of Ethnography at the Russian Academy of Sciences for her assistance in the collecting and processing of the material from the Smolyan territorial group.

The spread of the eastern complex in its higher values (from 47,1 % to 51,5 %) was traced to the Shiroka Luka region. In this region the highest per cent content of the proximal palmar triradius (71,6 %) was also established. In the Dospat-Devin region the higher values of the eastern complex is the result from the emergence of low values of the pattern intensity index; from the high frequency of the proximal palmar triradius; and from the low values of the true hypothenar patterns; and from the accessory interdigital triradii [2]. That is why the localization of the east-Europeoid component represented by the variations of the eastern complex is mainly concentrated in the west Dospat—Devin—Shiroka Luka region.

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Somatotypologic characteristics of gout patients — males

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44 gout male patients with sure diagnose (New York criteria), aged 44-55, have been studied. 11 anthropological features have been studied using the generally accepted classic methodics of Martin, Saller. The anthropometric data by S t. M u t a f o v et al. [7] and J a n e v et al. [8] for healthy Bulgarian population of the same age have been used for comparison. The data have been processed variation statistically. It has been found out that the differences in body massiveness dominate. Gout male patients are taller and have bigger body mass, chest width, pelvic width and torso length. These features are the basic elements of the endomorphic somatotype. The results are in agreement with literature data and our former results, according to which the gout is a disease definitely correlated with the endomorphic somatotype.

Key words: anthropology, physical development, somatometry, somatotype, gout.

Gout is one of the first diseases in connection with which constitutional predisposition is discussed [1, 2, 5]. The clinical experience, gathered in our country, in diagnosing and treatment of gout which becomes more frequent in Bulgaria as well as in other countries, directed us to investigate anthropometrically a group of gout patients [6]. According to literature data males of mature age suffer from it more often than women and the ratio varies from 4:1 to 20:1.

The aim of the present investigation is to study independently the biological information from anthropological features data, characterizing the somatotype of gout male patients.

An anthropometric investigation of 44 gout male patients with sure diagnose by the New York criteria aged 40—55, have been carried out. Most of them are watched over in the Clinics of Rheumatology (Sofia) and are periodically checked up.*

The metric data about 11 anthropological features as follows: main body characteristics (stature and weight), torso length, lengths of upper and low extremities, biacrominal circumference, sagital circumference, bicrystal circumference and three circumference features of body and extremities.

* The patients were committed for investigation by Assoc. Prof. Med. Dr K. Kanev, Assoc. Prof. Med. Dr T. Andreev and Med. Dr Bekjarova from the same Clinics.

Table 1. Comparative analyses of anthropological features in gout ill men and men from two control groups

Features	Gout	Control groups		T-criterion	
		Mutafov et al. [7]	Janev et al. [8]		
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$T_{[7]}$	$T_{[8]}$
Stature	171,83	169,05	168,55	2,81	3,45
Weight	91,58	77,30	74,10	5,79	7,24
Chest circumference	110,63	98,10	95,08	8,82	11,19
Biacrominal circumference	40,92	39,61	38,60	3,02	5,45
Sagital circumference	25,27	22,95	21,73	5,96	9,44
Bicrystal circumference	33,00	31,42	28,83	3,19	8,62
Arm circumference	32,33		29,75	—	6,22
Thigh circumference	58,73	—	53,63	—	6,61
Torso length	57,34	54,16	-	4,92	—
Upper extremity length	76,66	75,72	—	1,65	—
Low extremity length	94,44	95,02	--	0,86	—

The anthropometric data by St. Mutafov et al. [7] and Janev et al. [8] for healthy Bulgarian population of the same age have been used for comparison.

The data have been processed variation-statistically. The reliability of the established differences has been verified by Student's T-criterion at significance level $p < 0,01$. The results are given in Table 1.

The comparative analysis of the anthropometric differences between gout male patients and two healthy control groups shows that the body volume differences dominate.

Statistically reliable differences are established for the stature, weight and chest circumference (Fig. 1). The gout male patients are with pronounced higher stature (171,83 cm) compared to the two male control groups (169,05 cm and 168,55 cm). The gout male patients are significantly heavier (91,54 kg) comparatively to the healthy males (77,30 kg and 74,10 kg). The men examined by us are with more massive chest. Their chest circumference is 110,63 cm, and the two control groups' 98,10 cm and 95,08 cm respectively.

Gout male patients have well developed shoulder girdle and their biacrominal diameter is 40,92 cm in comparison with the two control groups of healthy men — 39,61 cm and 38,60 cm (Fig. 2). The sagital diameter of chest for the investigated ill men is with 2-3 cm bigger 25,27 cm compared to the data by Mutafov et al. and Janev et al. control groups — 22,95 cm and 21,73 cm. The pelvis dimensions of the ill men are bigger — they have wider and more massive pelvis. Their bicrystal diameter is with 2,5 to 4,5 cm bigger comparatively to the two control groups. The differences are statistically significant.

The bitrochanterial diameter of the gout male patients is about 3 cm bigger than the one of the control group of Janev et al.

The lack of data for the control groups by Mutafov et al. and Janev et al. about all of the circumference features, investigated by us, allowed us to determine the

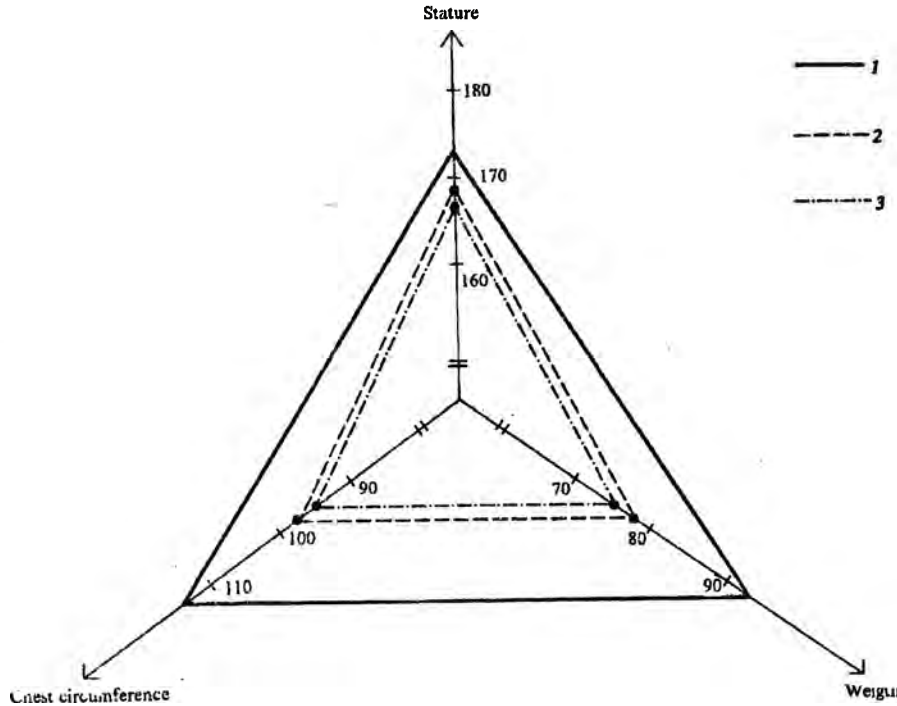

Fig. 1. Stature (cm), weight (kg) and chest circumference (cm) gout patients and two control groups of men
1 — gout; *2* — control (M u t a f o v et al. [7]); *3* — control (J a n e v et al. [8])

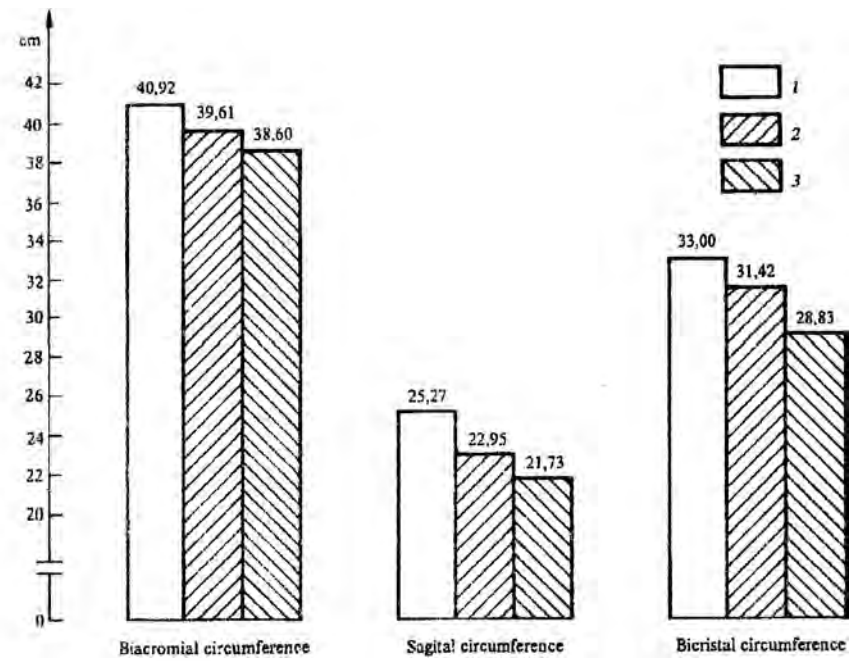

Fig. 2. Biacromial circumference (cm), sagital circumference (cm) and bicristal circumference (cm) of gout patients and two control groups of men
1 — gout; *2* — control (M u t a f o v et al. [7]); *3* — control (J a n e v et al. [8])

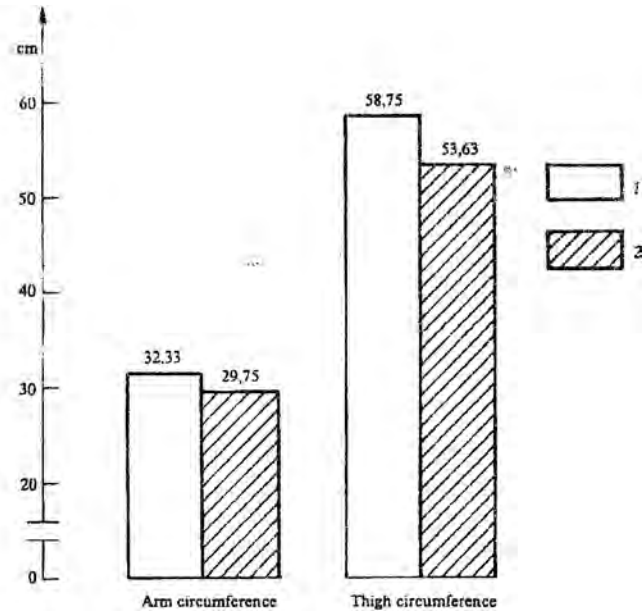

Fig. 3. Arm circumference (cm) and thigh circumference (cm) of gout patients and a control group of men
1 — gout; *2* — control (J a n e v et al. [8])

reliability of the statistical differences for the circumferences of arm and thigh only. The comparison with the data for healthy people by Janev et al. shows that the circumferences of arm and thigh of gout patients are with about 3 cm and 5 cm respectively bigger (Fig. 3). The established differences in circumference dimensions confirm the results obtained in the analysis of the data for the diameters of chest and pelvis, which show well developed shoulder girdle, massive chest and wide pelvi

From the data for the other anthropological features it is established that go patients have longer torso length comparatively to healthy men according to Janev et al. data.

In summary, the anthropometric status of the patients, investigated by us is characterized with big weight, long torso, massive chest and shoulder girdle and wide pelvis.

It can be said on the basis of the results obtained up to now, that even without constitutional diagnosis done to gout male patients, the data from directly investigated anthropometrical features allow to determine their somatotype as endomorphic. As it is known from out previous studies [3] and from the results of other authors who studied the constitutional predisposition towards certain diseases, the gout definitely is related with the endomorphic somatotype.

Practically, the results from the present study may be used in medicine practice without necessarily making constitutional diagnosis. The excessive fatness of family burdened endomorphic males is one of the risk factors provoking gout.

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Traumas of the postcranial skeleton in medieval population in Bulgaria

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Studies have been made of traumatic injuries and their complications in the postcranial skeletons of 3650 adults from 41 populations of Medieval Bulgaria. Most common are the fractures of the tubular bones of the upper and lower limbs, the clavicle and the ribs. About 50 per cent of the fractures had a favourable issue — no dislocations of fragments and no complications are observed. In 30 per cent of the fractures the bone fragments have healed up at an angle, with longitudinal dislocations. In 20 per cent of the traumas some traces of serious complications are observed, such as periostitis, ossifying fibrosis, post-luxational arthroso-arthritis, ankylosis, osteomyelitis, etc.

Key words: paleopathology, traumas, complications, Medieval Bulgaria.

The traces of various traumas found on the skeletal remains of people from different epochs play a distinctive role in the reconstruction of the way of life of ancient populations. Along with the group of degenerative-dystrophic lesions of the spine and the big joints of the limbs, traumatic lesions are among the most frequently observed pathological changes. Apart from common traumas related to the specific conditions of life and work, they also reflect the consequences of war engagements or heavy accidents.

The object of the present study are the traumatic injuries of the postcranial skeleton. They have been studied in 3650 adult individuals from 41 medieval necropoles on the territory of Bulgaria, dating from between the 8th and the 18th centuries.

The traces of various traumatic injuries have been studied in two directions:

1. Morphological changes, setting in due to the direct influence of the blow and in the process of healing.

2. Structural changes of the bones due to post-traumatic complications.

The excavated human osteal remains from all periods of the Middle Ages reveal distinct traces of various traumas. The peculiarities of the injured bone surfaces, as well as the localization of the lesion provide some evidence of the kind of weapon that was used, the strength of the blow, the course of the healing process, the attempted treatment and its outcome. Owing to the specific character of formation of the flat bones, the vertebrae and the tubular bones, there are differences in the way in which defects of the respective bones are manifested. The osteal traumas observed in medieval human remains can be classified, according to the type of weapon, as slashes, stabs,

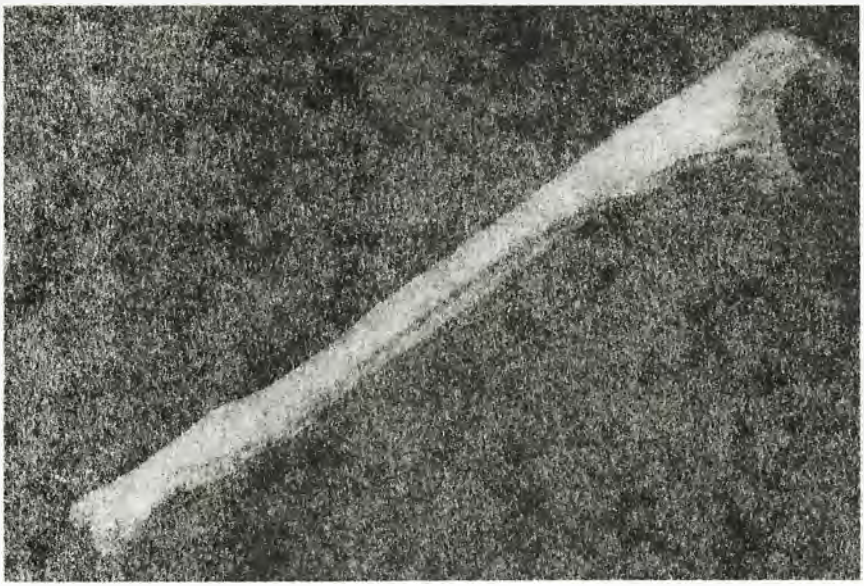

Fig. 1. Healed fracture of the distal part of the elbow, with a well formed callus

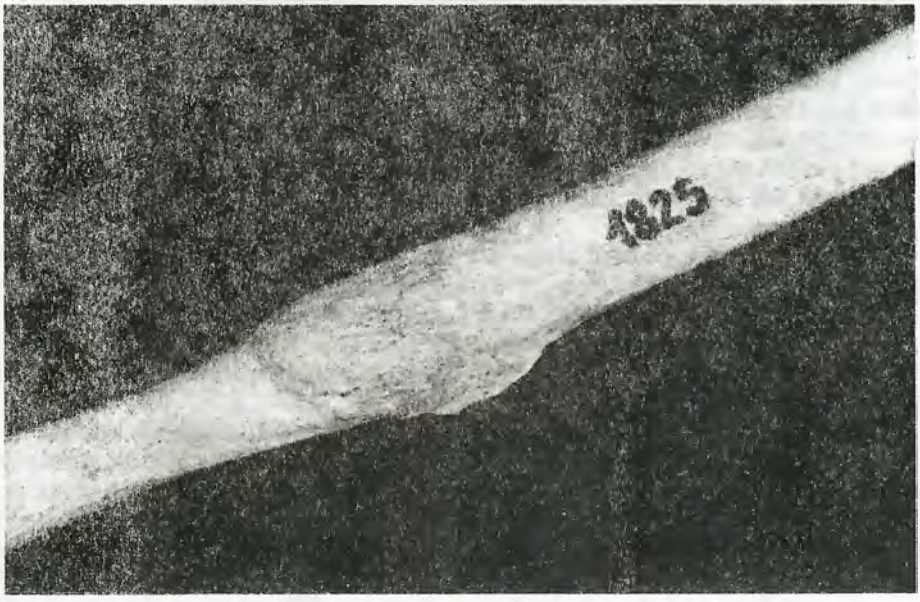

Fig. 2. Healed fracture in the middle of the elbow bone with an insignificant longitudinal dislocation of the fragments

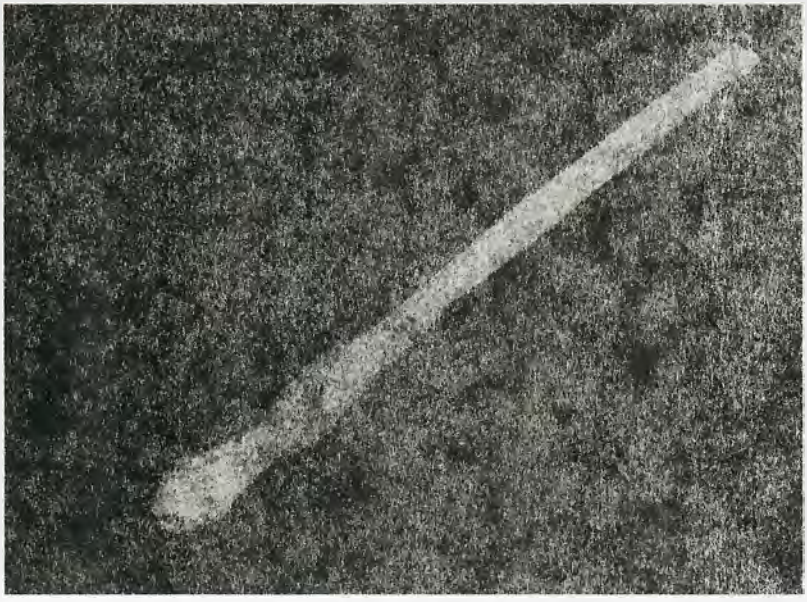

Fig. 3. Localized periostitis after a fracture of the distal part of a small leg bone

inflicted by cutting, chopping or blunt objects. Unlike cranial traumas in which the traces of slashes and stabs predominate, most frequent on postcranial skeletons are healed fractures inflicted with a blunt object. Bone lesions as a result of slash wounds are only observed in single cases, in the form of elongated defects with smooth edges, on the diaphysis of long bones.

According to their location, traumas are most frequently observed on the bones of the upper and lower limds (Fig. 1), the clavicles and the ribs. Considerably less frequent are traces from fractures of the pelvis or the vertebrae. Lesion frequency varies between 2,5 per cent and 8 per cent. A higher frequency and heavier traumas are commonly observed in the necropoles of medieval fortresses (Pernik [1], Odartsi [2], Jambol, Drustur [3], Bozhenishki Ourvich [4], Kavarna [5], Kaliakra); they can be accounted for by war engagements. In most cases the morphological changes in the structure of the affected bones testify to a favourable issue — the bone fragments have healed up with a well-formed callus and, most frequently, with insignificant, only longitudinal, dislocations (Fig. 2). This is the case with about 50 per cent of the fractures of long bones. At the same time, there is no atrophy of the affected bone, which suggests that the limb continued to function as usual. With some of those healed traumas a localized, irregular growth of the periosteum can be observed (Fig. 3). At times the healing of the bone edges was impeded, even in the absence of inflammatory complications or dislocations; in such cases the healing process has resulted in the emergence of the so-called pseudoarthrosis between the bone fragments, rather than in a callus formation. With combined fractures of the leg or the forearm, the formation of "neoarthrosis" [6] is observed between the two adjacent bones in the region of the fracture (Fig. 4). In those rare cases the movements of the newly formed joints, though extremely limited, burdened the adjacent joints and caused secondary degenerative changes. The second group of fractures consists of cases with pronounced dislocations. The bone fragments have healed up twisted, at an angle, or with a considerable longitudinal dislocation. This is the case with about 25 per cent of the

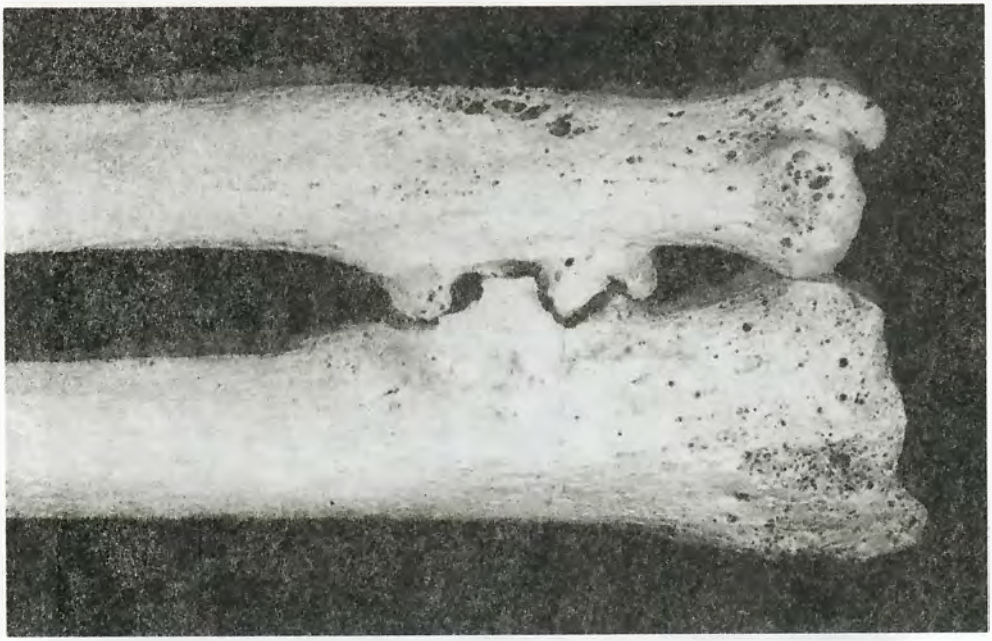

Fig. 4. "Neoarthrosis" after a trauma of the distal part of forearm bones

total number of fractures; with the majority of them the healing process went without any inflammatory complications, and the newly formed bone tissue has well enveloped the bone edges (Fig. 5). Such fractures are most frequently observed in the clavicles — the twisting and the longitudinal dislocation of fragments caused a pronounced deformation and shortening up to 3 cm. In rare cases the inexpedient treatment brought about a pronounced dislocation of the bone fragments (Fig. 6). The marked shortening and the anomalous healing impeded the functioning of the affected limb [7].

The third group of findings includes post-traumatic complications, such as periostitis, ossifying fibrosis, arthroso-arthritis, ankylosis, osteomyelitis, etc. Irrespective of the attempts to apply some treatment, in 20-25 per cent of the traumas of the postcranial skeleton some traces of slighter or more serious complications that emerged in the process of healing are observed (Fig. 7). Those were probably

Fig. 5. Fracture in the middle of a humeral bone, healed with angular dislocation of the fragments (X-ray)

Fig. 6. Deformation of the elbow bone after a fracture — a pronounced dislocation: angular, longitudinal and by twisting

open fractures with bruises and lacerations, fragmentational traumas and joint-injuries. A frequent manifestation of posttraumatic complications are the non-specific periostites (Fig. 8). An irregular growth of the periosteum is observed with combined fractures of the leg or the forearm; this sometimes leads to their knitting together. The periostites are usually localized but they can also diffuse through the bones. If there are torn muscle fibres or tendons, an ossification may set in of the layers of connective tissue in the muscles, as well as at the fastening points of the tendons. These are the so-called ossifying fibrosites.

Particularly serious post-traumatic complications are observed with fractures in the epiphyseal regions of the long bones of the limbs, accompanied by luxations or intraarthrous haemorrhages. In such cases the morphological changes usually affect both surfaces forming the joint. Among the changes observed are polishing and flattening accompanied by the formation of an additional joint-surface, as well as extensive exostosison the bone edges and heavy deformations (Fig. 9). Besides with traumas, such a morphological situation can be observed with congenital luxations of the coxo-femoral joint. Quite often the specific changes in the structure of the bone surfaces show that the degenerative changes were preceded or accompanied by inflammatory complications. Studying the numerous paleopathological findings from the Middle Ages, it should be noted that the majority of traumas affecting the joints were followed by complications of various gravity. Some of them resulted in ankyloses and caused disability. Particularly serious complications of this kind have been found among the population in the northern region of the Black Sea coast (Kavarna and Kaliakra — 15th-17th c.). In three cases, following a trauma of the knee joint, the lower limb was transformated into an immobile bone formation. With two of the traumas the ankylosation set in when the limb was in a stretched position (Fig. 10), and in the third case the bones knit at an angle. The morphological changes in the

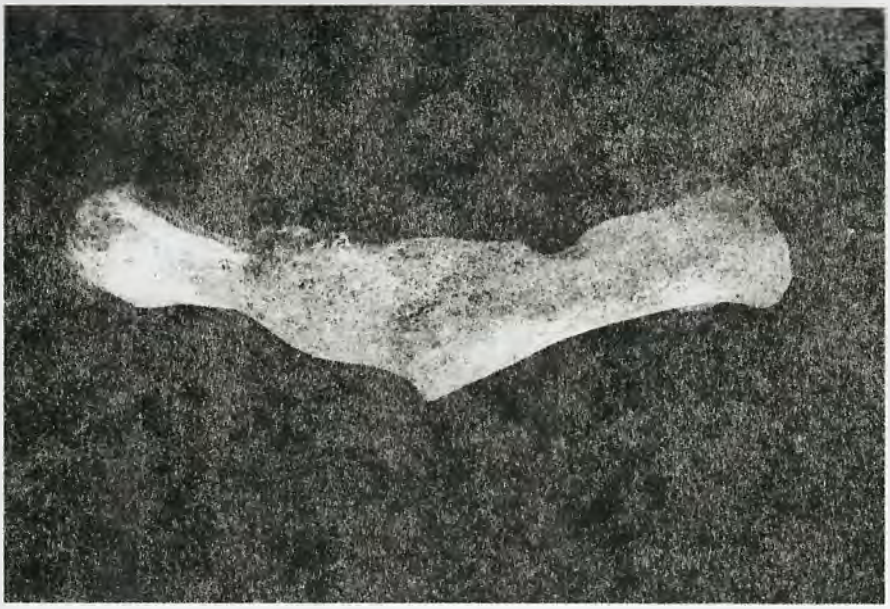

Fig. 7. Inflammatory complication after a fracture of the clavicle

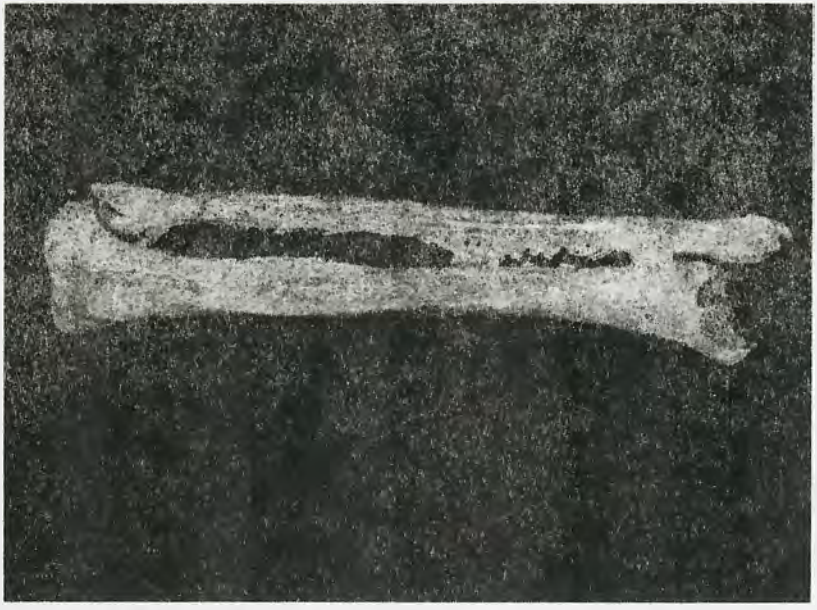

Fig. 8. Non-specific periostitis after a trauma of the leg

Fig. 9. Deforming arthroso-arthritis after a trauma of the elbow joint

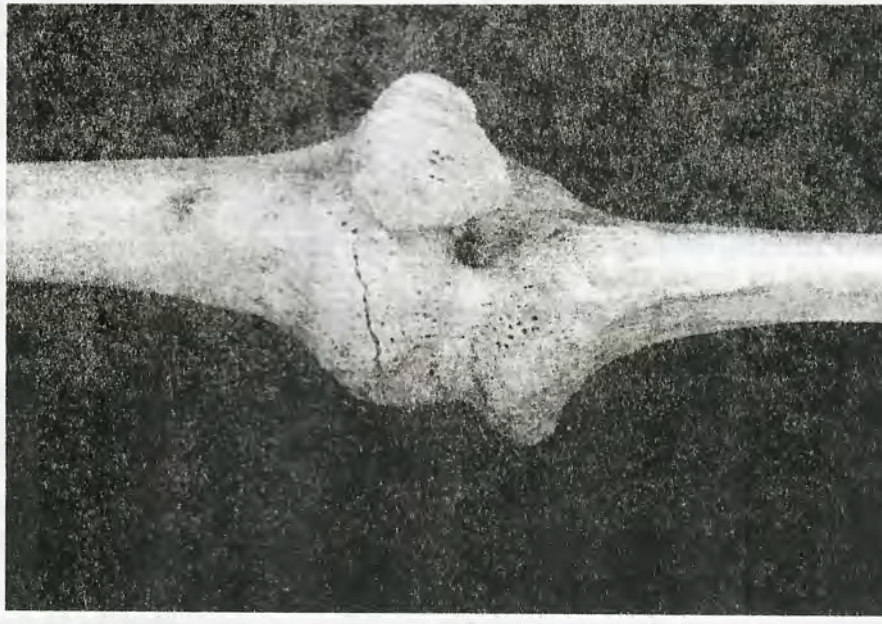

Fig. 10. Post-traumatic anchylosation of the knee joint

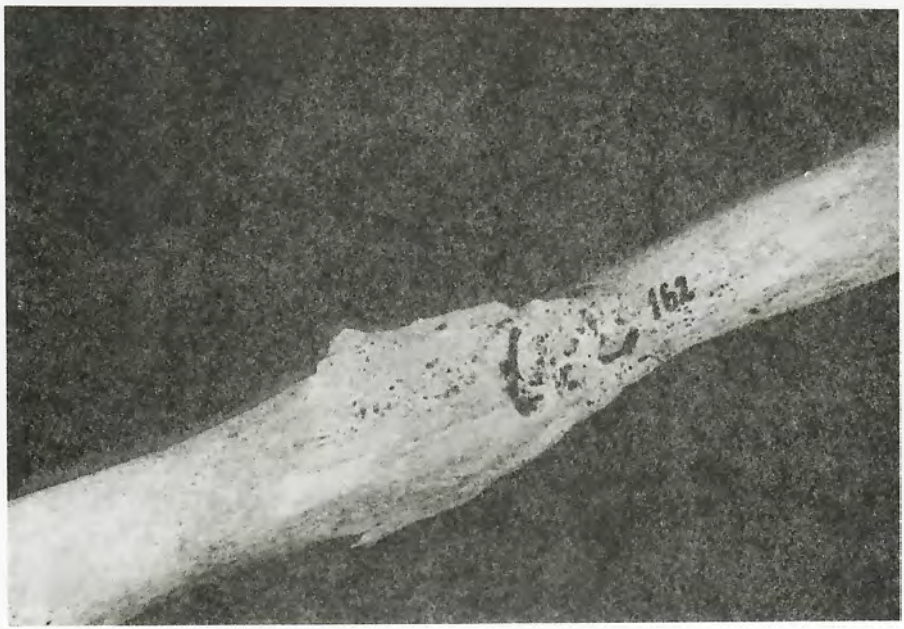

Fig. 11. Post-traumatic inflammatory process after a fracture of the femur

bone structure testify to a chronic inflammatory process, followed by secondary degenerative changes. Most probably, the frequent complications after joint-traumas were related to difficulties in the treatment of intra-arthrous haemorrhages whose impeded resorption caused injuries of the joint cartilage and the bone surface. Similar changes can occur as a result of repeated haemorrhages (usually in the knee joint) with haemophiliacs. In such cases there is no evidence of fractures or luxations. A characteristic morphological symptom is the enlarged distance between the two condyles in the distal end of the femur.

The other group of post-traumatic complications comprises the inflammatory processes affecting the diaphysis of the long bones. They occur with heavy fractures accompanied by considerable dislocations (Fig. 11) and open wounds. Sometimes the chronic inflammatory process is manifested by individual fistulisations; the typical manifestations of osteomyelitis (Fig. 12), with the formation of bone sequesters, are more rarely observed.

The traumas affecting the spine should be mentioned too. They are considerably less frequent — to some extent, owing to the more difficult preservation of affected vertebrae. Most often traces of indirect traumas can be observed — i.e. compressional fractures of vertebrae of the thoraco-lumbal section of the spine.

These are traumas that had a favourable issue but were followed by some degenerative changes in the form of osseous growths on the edges of the vertebrae (osteophytes of various sizes) and immobilisation of the affected section of the spinal column.

Summing up the results of the paleopathological study of the numerous osteal remains bearing traces of traumatic injuries, the following conclusions can be drawn:

1. In different periods of the Middle Ages the frequency of traumas affecting the postcranial skeleton varies from 2,5 % to 8 %. More numerous and morphologically more varied are the injuries of bones found in the necropoles of fortified towns; this is probably connected with frequent war engagements.

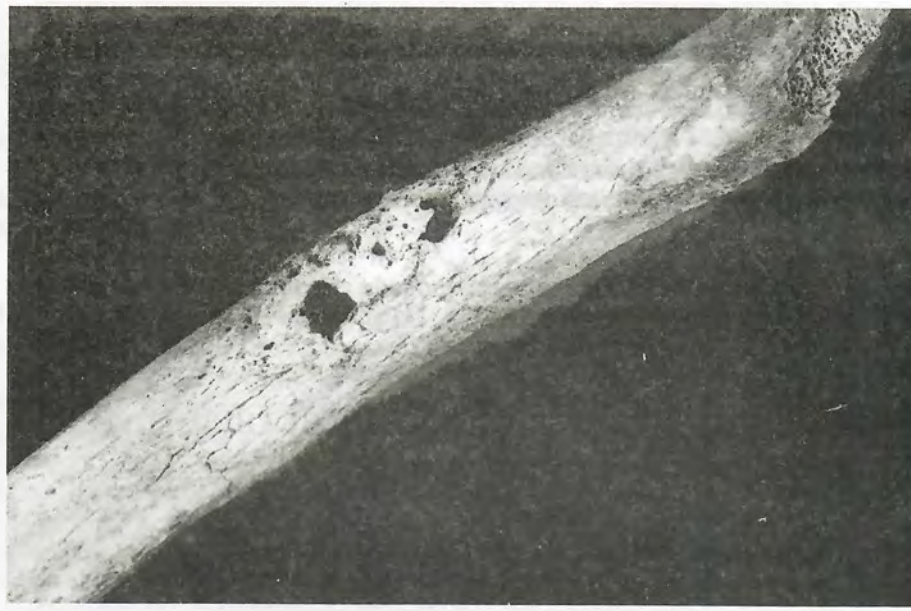

Fig. 12. Chronic osteomyelitis after a trauma of the femur

2. About 40-50 % of the fractures of long bones have a favourable issue — a well-formed callus and insignificant dislocation of fragments, which presuppose a medical help.

3. Characteristics of the epoch studied are the grave post-traumatic complication of joint-lesions.

4. The severe disability caused by some traumas and demanding prolonged care of the injured individual throws light on the social relationships in the population.

The above discussion makes it clear that the study of post-traumatic injuries traced on human skeleton of ages long past provides varied information both about the single individual and characteristic way of life of the whole population.

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Review Articles

Immunobiology of the mononuclear phagocytic system in central nervous system

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The different central nervous system (CNS) pathology is accompanied by "gliosis" — an increase in the number of the nonneuronal cells — astroglia, microglia, exogenous macrophages. Some of these cell populations are representants of the mononuclear phagocytic system. They are perhaps the least well-characterized in the brain. The origin, the role and their relations with other cell types remains a controversial subject. The importance of the mononuclear phagocytic system in immunopathology and development is essential but its mechanisms are unexplained.

Key words: Mononuclear phagocytic system, CNS, microglia.

Introduction

The microenvironment of the central nervous system (CNS) has mechanisms of the selfprotection against injury, infection and immunologically unknown substances. The blood brain barrier makes this microenvironment partially privilege immunological region. Some of the elements of the mononuclear phagocytic system (MPS) — exogenous for the brain macrophages, perivascular cells, supraependymal cells, and different types of microglia can be easily found in the brain. Especially microglia is also one of the CNS glial cell types with controversial function, but strongly characterized as a class of MPS [13, 21, 22]. All these cells, commonly named "brain macrophages" are considered to play an active role in a variety of neurological diseases [12, 14]. Their functions in forming a network of immunocompetent cells within the CNS is supposable but not clear till now

Cell types of the mononuclear phagocytic system in the brain and its origin

Now the most important cellular forms of MPS can be defined as follows [4]:

ameboid microglia in white matter perinatally;

— ramified microglia in grey and white matter postnatally;

— activated microglia in areas of secondary reaction after nerve transection and CNS inflammation;

— reactive (phagocytic) microglia in areas of trauma, viral infection or neuronal degeneration;

— perivascular cells arround the small blood vessels and capillaries in the brain parenhyma with antigenpresenting and phagocytic functions (belonging to the resident microglia [4]);

— supraependymal cells.

The group of mononuclear phagocytic system in CNS is probably completed by ED2 — positive perivascular cells [11, 17], specialized phagocytic type with bone-marrow origin. In the same group we can include subependymal and epyplexus cells.

It's well known that the cells of the MPS in the brain are of multiple and different origin. The classical concept of del Rio Hortega (1932) [3] is in support of the cell unity of the MPS in CNS. This concept states that mesodermal elements invade the mammalian brain near the birthtime and remain scattered in the CNS throughout life, representing a source of microglia and macrophages in pathologic conditions [9]. Some authors suggest a blood-monocytic origin for ameboid microglia but the majority of resting microglial cells are of local, presumably neuroectodermal origin. Another conclusions [23] state that the normal adult CNS contains no cells belonging to the monocyte — macrophage lineage. But the majority opinions are that microglia belong to the mononuclear phagocytic system and originate from the blood monocytes-macrophages that invade the CNS in embrionic development [1, 4, 18]. Now there is agreement that microglia is a distinct class of MPS [7].

Characteristics and morphology of the MPS in the brain

Described under different terms the subpopulations of the MPS in CNS have a morphology very similar to other brain cells both in vivo and in vitro. Morphologically to make the difference is possible only between the ramified (resting, "quiescent") and the other cells of this system.

Microglia comprises between 5 and 20 % of the total glial cell in brain. Intercellular membrane contacts among microglia or between microglia and other neural cells have not been described in normal CNS [12]. Immunochemistry (monoclonal antibodies and lectins), enzyme histochemistry, metal impregnation and electron microscopy have been used to identify microglia. The resident microglia represents a major source of endogenous brain macrophages [8] and is thought to be involved in antigen presentation in vivo in CNS by virtue of the fact that they express major histocompatibility complex (MHC).

So-called "ameboid" microglia appears in the brain during the late stage of embriogenesis. These cells, with phagocytic properties disappear in postnatal period [2]. Morphologically they have been characterized by being round in shape and by exhibiting varying numbers of cytoplasmic vacuoles. They show high levels of lysosomal enzymes: acid phosphatase and non-specific esterase [5]. The "ramified" microglia appears pleomorphic, elongated or triangular with round or oval nucleus. The cells possess abundant, thin branches of cytoplasm, and contain a few primary lysosomes and varying numbers of secondary lysosomes.

In contrast to peritoneal macrophages microglia divides in culture [6]. Microglia exhibits functional characteristics common to cells of MPS: phagocytosis, Fc and CR receptors, MHC antigen expression, antigen presentation, interleukin-1 synthesis,

tumour cytotoxicity, superoxyde anion production and responsiveness to colony-stimulating factor [10]. The supraependymal cells have the phagocytic abilities of macrophages. The study of the other cell types of MPS in brain — subependymal and epyplexus cells is restricted by the absence of the procedures of their isolations.

It's clear that there are many difficulties in distinguishing all these populations — all of them possess a dendritic morphology and they are immunocompetent and phagocytic. They share features both of peritoneal macrophages and of the glial cells [6].

Cell markers and immunophenotype

For microglia the specific visualization with RCA-1 (*Ricinus communis* agglutinin 120) and GSA I-B4 (*Griffonia simplicifolia* B4 isolectin), widely used in histochemistry, are based on the presence of membrane associated glycoconjugates containing terminal α-D-galactose residues. A specific marker for ameboid microglia in vitro and ramified microglia in vivo, based on the phosphotyrosine immunoreactivity is proposed [26]. Another specific marker — OX-6 antigens, characteristic for the antigen-presenting cells in common is used for detection of activated microglia. The regional differences in the number of MHC class II antigen-presenting microglial cells in normal animals (OX-6 positive cells) correspond with the preferential sites of the eventual inflammatory infiltrates founded in EAE [27].

The OX-42 monoclonal antibody is specific for both ameboid and ramified microglia as well as for the cells of monocyte/macrophage lineage [20]. In the same time the OX-41 antibody labels only monocyte/macrophage lineage and not microglia [9]. This marker may be useful in discriminating microglia in CNS from blood-born phagocytes which invade the brain in pathologic lesions.

In tissue sections the ED1, ED2 and ED3 monoclonal antibodies label perivascular cells but not microglia. It is very interesting fact that relatively late in the course of EAE microglial cells in CNS are found to express ED1 antigen in their cytoplasm and this expression is probably a sign of phagocytic activity [10]. Common markers from this group for microglia and monocyte/macrophage cells are only ED7 and ED8. Minor differences in positive microglia markers are found between cultures derived from developing and mature central nervous tissue [10].

Discussion

As shown above it can be expected that the cells of MPS play a crucial role in the development and in the brain pathology. The experiments show a possible transformation of ameboid microglia into ramified during postnatal development. The term functional plasticity [24] refers to changes in microglial morphology, immunophenotype and functions that occur as the cells are confronted with a changing microenvironment, which will be result of pathology or normal development [25]. Microglia is implicated in wound healing, regulation of astrocytic differentiation, immune response and amyloid deposition in Alzheimer disease [20]. Whole specific cell pool of MPS in the brain plays an important role as immunological effector cells [19].

Activated microglia shows ameboidal shapes and has similar functions as monocytes-macrophages [21]. Ameboid microglia serves as principle scavenger cells during the development of CNS but during postnatal development it differentiate into ramified microglia, forming with the help of the other mononuclear phagocytes a potential immunoeffector network in adult brain [24].

Diseases where an active role for MPS has been proposed include AIDS [15], Alzheimer's disease and Multiple sclerosis [12].

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