

TOMUS XXXVI

FASCICULUS 6

1990

FOBLAN 36 (6) 273-352 (1990)

Folia biologica

JOURNAL OF CELLULAR
AND MOLECULAR BIOLOGY

ACADEMIA SCIENTIARUM
BOHEMOSLOVACA



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ISSN 0015-5500

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Phagocytosis of Synthetic Particles in Earthworms. Effect of Antigenic Stimulation and Opsonization

(*Lumbricus terrestris* / phagocytosis / opsonization / synthetic particles / antigenic stimulation)

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Received March 22, 1990

Abstract. We examined the phagocytic activity of coelomocytes in earthworms *Lumbricus terrestris*. The synthetic polymeric HEMA particles were used in this study. The phagocytic capacity was increased after the protein administration or preincubation of HEMA particles with coelomic fluid. Furthermore, we isolated and characterized the proteins adsorbed on the surface of the particles which have opsonin-like properties.

Phagocytosis, the recognition and ingestion of solid material by cells, has been the subject of detailed investigations since the pioneering work of Metchnikoff (1887). During the process of evolution phagocytosis developed into an important mechanism of immune defense. Although many cells demonstrate some degree of phagocytosis, only a few types of them exhibit this ability in an active fashion. All types of coelomocytes of *Lumbricus terrestris* were found to be phagocytic, with the exception of chloragogen cells (Stein et al., 1977). In vertebrates, the ingestion of foreign materials by phagocytic cells may be facilitated by humoral factors (opsonins) that coat these materials. Only little is known about a dependence of phagocytosis on humoral factor in earthworms (Stein and Cooper, 1981). However, some results suggest that several components of *L. terrestris* coelomic fluid possess common functions with human complement (Laulan et al., 1983, 1988). It is known that earthworms are able to respond to stimulation by a protein antigen by enhanced proteosynthesis and antigen-binding capacity of coelomic fluid proteins and coelomocytes (Tučková et al., 1988). Therefore we have tried to determine the influence of the stimulation by a protein antigen or opsonization of HEMA particles on phagocytosis of coelomocytes *in vivo*.

MATERIALS AND METHODS

Animals

Adult earthworms, *Lumbricus terrestris*, were used and kept at 15°C in soil.

Stimulation

Earthworms were stimulated by injection of arsanilic acid (Hopkins and Williams, Essex) coupled to human serum albumin (approximately 20 : 1

Abbreviations: HEMA particles — 2-hydroxyethylmethacrylate particles, ARS-HSA — arsanilic acid coupled human serum albumin, LBSS — *Lumbricus* balanced salt solution.

ratio; ARS-HSA; Ingraham, 1951) in 3% agar gel (10 μ g in 20 μ l per animal) into the coelomic cavity.

Phagocytosis of HEMA particles

The preparation and characteristics of 2-hydroxyethylmethacrylate copolymer particles (HEMA) have been detailed elsewhere (Větvička et al., 1982; Fornůsek and Větvička, 1986). Suspension of HEMA particles (10 μ l, 10^7 /ml) in *Lumbricus* balanced salt solution (LBSS, Stein and Cooper, 1981) was injected into the coelomic cavity. The coelomic fluid with coelomocytes was obtained by puncturing the coelomic cavity with a Pasteur pipette after 3 h. Non-adherent HEMA particles were removed by washing in LBSS. Cytological preparations were stained with May-Grünwald-Giemsa and examined with a light microscope.

Opsonization

Suspension of HEMA particles (10^7 /ml) was mixed with an equal volume of cell-free coelomic fluid or mouse serum. After the incubation for 1 h at 20°C or 37°C the suspension was washed and injected as described above.

Elution of opsonizing proteins

Proteins adsorbed on the surface of HEMA particles preincubated in coelomic fluid (as described above) were eluted with glycine-HCl buffer (pH 2.4; 0.018 M), concentrated by ultrafiltration, and analysed by SDS-PAGE.

Polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS-PAGE)

SDS-PAGE was performed on 10% gel (Laemmli and Favre, 1979). Separated proteins and molecular weight markers (Pharmacia, Uppsala, Sweden) were stained with Coomassie Blue R250.

Statistics

Student's *t*-test was used to statistically analyse the data.

RESULTS

Differential counts of coelomocytes

Control differential counts of coelomocytes are presented in Table 1. We observed changes in the percentage of granulocytes, acidophils (increase), and basophils (decrease, Table 1) after the introduction of synthetic particles into the coelomic cavity. Furthermore, the differential counts were influenced by an antigenic stimulation (ARS-HSA; Fig. 1). Four days after the stimu-

Table 1. Differential counts of coelomocytes of *L. terrestris* (mean $\pm$ SD).

% of	Control animals	During phagocytosis
Basophils	56.1 $\pm$ 5.7	32.7 $\pm$ 2.9
Granulocytes	12.0 $\pm$ 4.4	28.2 $\pm$ 3.1
Acidophils	9.3 $\pm$ 3.9	18.7 $\pm$ 1.3
Neutrophils	22.6 $\pm$ 4.9	20.4 $\pm$ 3.4

Nonopsonized particles were used for phagocytic test.

lation, increased counts of granulocytes were seen and the percentage of basophils decreased. No significant changes in the numbers of neutrophils and acidophils were observed. Intracoelomic injection of LBSS or agar did not cause any alterations in differential counts. Since we did not detect intensified incorporation of [3 H] thymidine into coelomocytes after the stimulation (unpublished studies), we cannot determine whether the changes in differential counts are caused by proliferation or by the recruitment of coelomocyte subpopulations from another cell compartment.

Phagocytosis

We found that granulocytes are the main type of phagocytizing cells; acidophils and basophils are opportune phagocytes, and phagocytized material was rarely seen in neutrophils (Plate XLIII, Fig. 2). The maximum phagocytic activity we observed on the fourth day after the stimulation by the antigen (Fig. 3). If we take the changes in differential counts into consideration, the phagocytic activity of granulocytes was decreased, but the percentage of phagocytic granulocytes from the whole population of coelomocytes has reached the maximal value on day 4 after the stimulation (Fig. 1). On the other hand, the phagocytic activity of basophils was increased by the antigenic stimulation. Phagocytosis of coelomocytes from animals treated with LBSS was approximately equal to the control level (Fig. 3).

Opsonization

The phagocytosis of HEMA particles preincubated for 1 h with cell-free coelomic fluid from nonstimulated and stimulated (ARS-HSA) *L. terrestris* is approximately twice higher in comparison with controls (Fig. 4). The numbers of engulfed preincubated HEMA particles per cell are so large that an evaluation of phagocytic index is not possible (compare Plates XLIII, XLIV, Fig. 2 and 5). Furthermore, the phagocytic activity of basophils was significantly increased when the HEMA particles preincubated with coelomic

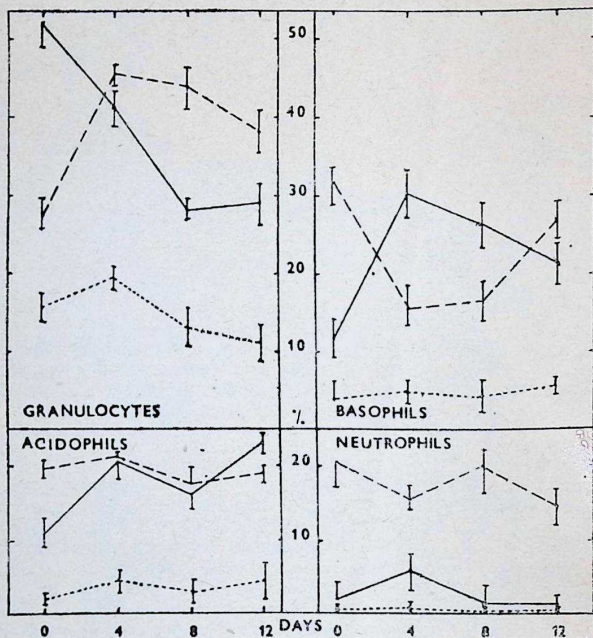


Fig. 1. Changes in differential counts (---), phagocytic activity (—), and phagocytosis in the whole coelomocyte population (....) after stimulation by ARS-HSA. Indicated means $\pm$ SD.

fluid from stimulated animals were used. We have detected a similar effect, albeit not so striking, when HEMA particles have been preincubated for 1 h with mouse serum.

Characterization of opsonizing proteins in coelomic fluid

In order to characterize proteins of coelomic fluid which adsorbed on HEMA particles during the incubation (for 1 h at 20°C), elution with glycine-HCl

buffer was performed. SDS-PAGE analysis of eluted proteins showed the presence of two opsonizing proteins in coelomic fluid with molecular weight 62 kDa and 68 kDa (Plate XLV, Fig. 6).

DISCUSSION

The main aim of our paper was to study the *in vivo* phagocytic activity of coelomocytes in earthworms *Lumbricus terrestris*. Differential counts of coelomocytes isolated from untreated animals were approximately equal to those described by Cooper and Stein (1981), but during the first 3 h after the injection of HEMA particles into the coelomic cavity they were significantly altered (Table 1). Rapid changes in differential counts and relative low proliferative activity (6.7% of labelled cells detected by means of quantitative autoradiography) reflect that the major cause is presumably the recruitment of subpopulations of coelomocytes from another cell compartment and the migration of these cells. The differential counts were similarly affected by protein stimulation.

All subpopulations of coelomocytes are able to engulf synthetic HEMA particles. There are some experimental data on the phagocytic activity (Stein et al., 1977; Stein and Cooper, 1981; Laulan et al., 1988), but the evi-

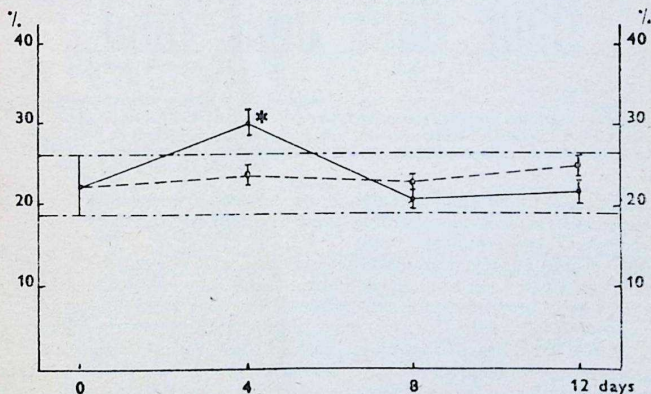


Fig. 3. Phagocytic activity of coelomocytes after stimulation by ARS-HSA (—) and LBSS (---). Horizontal lines represent control levels. Indicated means $\pm$ SD. * Significant difference at $P < 0.05$ level.

dence on phagocytosis of inert synthetic particles is lacking. The major phagocytic cell type are granulocytes, but their phagocytic activity decreased after antigenic stimulation. On the other hand, the percentage of positive basophils increased. Nevertheless, we can conclude that the *in vivo* phagocytosis of coelomocytes is enhanced by the antigenic stimulation (not by LBSS or agar). These results are in good agreement with the known fact

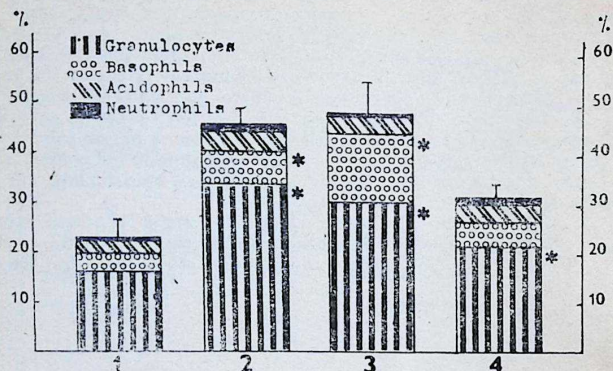


Fig. 4. Phagocytic activity of coelomocytes. 1, controls; 2, HEMA particles preincubated in coelomic fluid; 3, HEMA particles preincubated in coelomic fluid from stimulated earthworms; 4, HEMA particles preincubated in mouse serum. Differences of samples 2, 3, 4 are significant at $P < 0.05$ level. * Significant differences at $P < 0.05$ level for subpopulations.

that earthworms are able to respond to a protein antigen by enhanced proteosynthesis and antigen-binding capacity of coelomic fluid proteins and coelomocytes (Tučková et al., 1988).

Some authors suggest that several components of coelomic fluid possess common functions with human complement and potentiate the phagocytic activity of coelomocytes (Stein and Cooper, 1981; Laulan et al., 1983, 1988). In this report we describe twofold higher phagocytic activity of coelomocytes if the HEMA particles preincubated with cell-free coelomic fluid are used. Furthermore, the phagocytic index of coelomocytes was increased, albeit was not determined in spite of the large amount of engulfed particles inside the cells. The increase of phagocytic power in the case of HEMA particles pretreated with phylogenetically distant mouse serum was not so striking, but cannot be overlooked. This is particularly interesting, as no effect of

opsonins on the phagocytosis of HEMA particles has been observed in a mammalian model (Holub et al., 1984). On the basis of these results we suppose that enhanced phagocytic activity of coelomocytes may be caused by binding of some coelomic fluid proteins on the surface of HEMA particles and that the levels of these proteins are influenced by protein stimulation. These findings are in good agreement with our previous report (Bilej et al., 1990) in which we have presented a flow cytometric study of *in vitro* phagocytic activity of coelomocytes of cognate species *Eisenia foetida*. We have observed that some coelomocytes are able to respond, on *in vivo* protein stimulation or on opsonization, by enhanced phagocytic activity and index. It is hypothesized that earthworm's coelomocytes might recognize a different type of opsonins than vertebrate cells. However, we isolated two proteins (62 kDa and 68 kDa) adsorbed on HEMA particles, their biochemical properties and the exact mechanism of their action are now intensively studied in our laboratory.

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Фагоцитоз синтетических частиц у дождевых червей

М. Билей, В. Ветвичка, Лудмила Тучкова, И. Требихавски, М. Коукал, П. Шима

Резюме. В работе описан фагоцитоз полимерных НЕМА частиц целомоцитами дождевого червя (*Lumbricus terrestris*). Фагоцитарная активность клеток повышается после стимуляции антигенным протеином, а также после преинкубирования НЕМА частиц в целомовой жидкости. Опсонизирующие факторы целомовой жидкости, адсорбированные на поверхности частиц, были выделены и охарактеризованы путем гель-электрофореза.

Serological Analysis and Mixed Lymphocyte Reactions of Cells Carrying a Spontaneous H-2^{bm25} Mutation*

(major histocompatibility complex / mixed lymphocyte culture)

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Received April 17, 1990

Abstract. Serological analysis of mutant H-2^{bm25} cells revealed that the bm25 mutation in the K^b region of the H-2 complex occurred at a site corresponding to the specificity H-2.39. Monoclonal antibody HB 41 (anti-H-2.m39) did not react to mutant bm25 cells in the direct cytotoxicity test whereas the reaction to standard B10 cells was not changed. B10.RIII-H-2^{bm25} cells retained some absorption ability however and were capable of removing partially the anti-H-2.39 antibody in absorption tests. Cells bm25 responded in mixed lymphocyte reactivity (MLR) assay by proliferation to stimulating standard B10 cells, but in the opposite direction no positive MLR was observed.

H-2 mutations are a suitable tool for studying the fine structure of the major histocompatibility complex and for elucidating pleiotropism of H-2 genes. Recently the changes, which are developed by H-2 mutations, can be made clear on molecular level and it can be exactly determined which amino acids are changed in the mutant molecule in comparison with standard type of antigen. The most commonly used method for detecting H-2 mutations is the skin grafting (Klein, 1978; Kohn et al., 1978). In this way a new H-2 mutation, designated bm25 (Mouse News Lett. No. 84, 1989), has also been found. The bm25 mutation was observed among the animals of (B10 × B10.RIII) Bc3 generation and transplantation analysis has shown that the mutation occurred in the H-2^b haplotype of C57BL/10SnY (B10) strain (Blandova and Ratkin, 1987). Using complementation tests with H-2 recombinant strains the bm25 mutation has been located to the H-2K^b region (Blandova and Ratkin, 1987). This mutation is carried by strain B10.RIII-H-2^{bm25}.

The aim of the study was directed toward serological analysis of the membrane product of H-2^{bm25} mutation and its examination in mixed lymphocyte culture.

MATERIALS AND METHODS

Mice

The breeding nucleus of B10.RIII-H-2^{bm25}/Y (bm25) mice was obtained from the Research Laboratory for Experimental Biological Models, Academy of Medical Sciences of the USSR, Moscow Region. All other inbred strains —

Abbreviation: MLR — mixed lymphocyte reactivity.

* Dedicated to the memory of Dr. Zinaida Konstantinovna Blandova, scientific worker of Research Laboratory for Experimental Biological Models, Academy of Medical Sciences of the USSR, who was engaged in the investigations of H-2 mutations and was our colleague in this field.

C57BL/10ScSn (B10), B10.M, BALB/c and B10.A — are maintained at the Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Praha.

Antibodies

Polyclonal anti-H-2 antisera of the ASP series prepared in the Institute of Molecular Genetics, Praha, and monoclonal anti-H-2 antibodies produced by Ozato and Sachs (1981) were used. Polyclonal antisera and monoclonal antibodies were chosen, so that they reacted with the H-2^b haplotype from which the bm25 mutation was derived. H-2 specificities of all used antibodies are given in Table 1 and were confirmed by testing against a panel of inbred strains with relevant H-2 haplotypes.

Microcytotoxicity test

Microcytotoxicity test was carried out according to Pincus and Gordon (1971), with some modifications. Suspensions of lymph node cells in RPMI 1640 medium (5×10^6 cells/ml) were incubated with antiserum or monoclonal antibody and nontoxic rabbit serum (source of complement) in microplates at 24°C for 60 min. The proportion of dead cells was determined after eosin staining. Details are given in a former paper (Čapková et al., 1983).

Absorption *in vitro*

Washed packed spleen, thymus and lymph node cells were mixed with an equal volume of antiserum or monoclonal antibody and incubated for 120 min at 4°C with occasional shaking. After incubation the cells were spun down and the supernatant was tested for residual antibody activity in the microcytotoxicity test.

MLR assay

Suspensions of mixed spleen and lymph node cells (roughly 1 : 1) were prepared in RPMI 1640 medium supplemented with antibiotics (penicillin, streptomycin), glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 10% foetal calf serum. A 0.1 ml amount of reactive cells (2.5×10^6 cells/ml) and 0.1 ml of stimulator cells (5×10^6 cells/ml) were pipetted into individual wells of 96-well flat-bottomed tissue culture plates (Sterilin Ltd., Feltham, England). Before pipetting into microplate wells stimulator cells were treated 30 min at 37°C with mitomycin C (50 µg/ml). The cells were incubated at 37°C in an atmosphere of 5% CO₂ in air for 4 days and 1 µCi [³H] thymidine was added to each well for the final 18 h of culture. Cells were harvested onto glass fibre filters with a multiple sample harvester. The incorporated activity was measured in a Beckman liquid scintillation counter.

Table 1. Cytotoxic reactivity of B10 and B10.RIII-H-2^{bm25} lymph node cells with anti-H-2 polyclonal and monoclonal antibodies

ASP, No.	Polyclonal antibodies H-2 specificity	Cytotoxic titre ⁻¹ * with:	
		B10	B10.RIII-H-2 ^{bm25}
98	K ^b (33, 39, 53)	640	320
208	K ^b (5, 33)	160	160
215	K ^b (5, 33)	640	640
235	K ^b (33, 39)	640	320
238	K ^b (5, 33)	2560	1280
251	K ^b (33, 39)	2560	640
289	K ^b D ^b (2, 33, 46)	1280	1280
305	D ^b (2)	80	80
308	D ^b (2, v _w 56)	320	320
309	K ^b D ^b (2, 33, 56)	160	160
343	D ^b (2, 27, 28)	320	320
349	K ^b (33, 39)	2560	2560

Monoclonal antibodies		Cytotoxic titre ⁻¹ * with:	
Designation	H-2 specificity	B10	B10.RIII-H-2 ^{bm25}
K9 178**	K ^b	1280	0
HB 19**	D ^b	5120	5120
HB 35	I-A ^b (Ia.8)	160	320
HB 38	I-A ^b (Ia.20)	80	40
HB 41**	K ^b (H-2.m39)	1280	0

* Cytotoxic titre⁻¹ is the reciprocal value of the highest serum dilution with more than 50% killed cells.

** 1:5 diluted ascites.

RESULTS

In serological analysis of the bm25 mutation, the mutant cells B10.RIII-H-2^{bm25} and standard cells B10 were first examined with a battery of H-2 antisera (Table 1, Part 1). In the direct cytotoxicity test, no qualitative difference in the reactivity of mutant and standard cells to conventional anti-H-2^b antisera was found. All the H-2 antisera reacted with both types of cells.

Nevertheless, some conventional H-2 antisera, particularly those containing antibodies against the specificity H-2.39 (ASP 98, 235, 251) reacted to bm25 cells more weakly than to B10 cells. The weaker cytotoxic reactions could indicate a quantitative difference but they could with respect to the antibody specificity of the antisera used also reflect a qualitative difference in H-2 antigens between mutant and standard cells.

Table 2. *In vitro* absorption of anti-H-2^b antiserum ASP 235 (anti-H-2.33, 39) and monoclonal antibody HB 41 (anti-H-2.m39) with B10 and B10.RIII-H-2^{bm25} cells

Antiserum, MonoAb	Reciprocal of cytotoxic titre* with cells		
	B10	B10.M	B10.RIII-H-2 ^{bm25}
ASP 235	640	80	320
ASP 235 abs.B10	16	0	8
ASP 235 abs.B10.RIII-H-2 ^{bm25}	32	8	4
HB 41**	320	320	0
HB 41 abs.B10	8	4	0
HB 41 abs.B10.RIII-H-2 ^{bm25}	32	32	0

* Cytotoxic titre is the highest dilution of the antiserum or monoclonal antibody at which more than 50% of the target cells were killed.

** HB 41 monoclonal antibody was 5 × diluted.

Moreover, in the direct cytotoxicity test bm25 and B10 cells were tested against anti-H-2^b monoclonal antibodies directed against products of different regions of the H-2 complex (Table 1, Part 2). Two of the five monoclonal antibodies used, K9 178 (anti-K^b) and HB 41 (anti-K^b, anti-H-2.m39), distinguished strictly bm25 and B10. Both antibodies showed strong reactions to standard cells and no reaction to bm25. From a negative reaction of bm25 cells with HB 41 antibody, the specificity of which precisely corresponded to anti-H-2.39, we concluded that on the H-2K^b molecule of the bm25 mutation a site (epitope) corresponding to the specificity H-2.39 was changed. This conclusion was verified in a further step by absorption tests.

In absorption tests we used antiserum ASP 235 (anti-H-2.33, 39) and monoclonal antibody HB 41 (anti-H-2.m39). The antiserum and antibody were absorbed with both mutant and standard cells and then tested with cells

Table 3. Antiserum production between the mutant bm25 and parental B10 strain or its F₁ hybrids

Immunization*	% Lysis**
B10.RIII-H-2 ^{bm25} anti B10	0
B10 anti B10.RIII-H-2 ^{bm25}	0
(B10 × C3H)F ₁ anti B10.RIII-H-2 ^{bm25}	0
(A.CA × B10)F ₁ anti B10.RIII-H-2 ^{bm25}	0

* Immunizations were performed by 10 weekly injections of thymus, lymph node and spleen cell suspension (one donor per 10 recipients) and bleeding was carried out 1 week after the last injection.

** The cytotoxicity of sera was tested on lymph node cells.

used for absorption and with B10.M cells of the H-2ⁱ haplotype that are H-2.39 positive and H-2.33 negative. The results are summarized in Table 2. As expected, B10 cells were capable of effectively absorbing antibodies from antiserum ASP 235: they absorbed completely the reaction against B10.M (anti-H-2.39 antibody activity) and substantially weakened the reaction to B10.RIII-H-2^{bm25} (titre 1/8) and B10 (titre 1/16). These residual reactions corresponded to the anti-H-2.33 antibody which was not completely removed by absorption.

In the case of absorption of antiserum ASP 235 by mutant B10.RIII-H-2^{bm25} cells we expected that bm25 cells, as a result of their changed H-2.39 specificity, would not be capable of absorbing the anti-H-2.39 antibody and that the reaction to B10.M would be retained. However, bm25 cells significantly decreased the activity of antiserum ASP 235 against both B10 (titre 1/32) and B10.M (titre 1/8). This suggests that they retained some capacity to absorb also the anti-H-2.39 antibody. Similar results were obtained with absorption of antibody HB 41 with B10 and B10.RIII-H-2^{bm25} cells. Not only B10, but also B10.RIII-H-2^{bm25} cells that did not react with

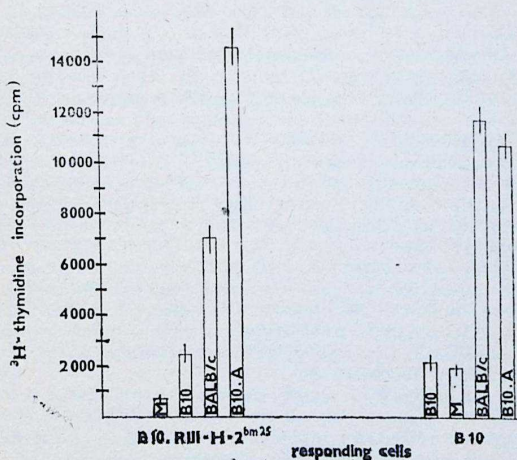


Fig. 1. Graphic representation of mixed lymphocyte reactions of mutant B10.RIII-H-2^{bm25} and standard cells. M — mutant cells, cpm — counts per minute.

antibody HB 41 in the direct cytotoxicity test were capable of absorbing the anti-H-2.m39 antibody irrespective of having their absorption ability reduced in comparison with standard cells.

In studying the bm25 mutation we also tried to prepare antibodies by reciprocal immunization between the mutant and the standard B10 strain or its F₁ hybrids. The results were negative (Table 3). After immunization no antibodies were demonstrated in the reciprocal strain combinations.

Mutation bm25 was further tested in mixed lymphocyte culture. Both mutant bm25 and standard B10 cells were used as stimulators and responders, and their mutual reactivity is shown in Fig. 1. A positive mixed lymphocyte reactivity (stimulation index of 3.5; the stimulation index = cpm in culture of cells responding to allogeneic cells : cpm in culture of cells responding to syngeneic cells) was noted in the mutant-standard strain combination where B10.RIII-H-2^{bm25} cells were the responders. In the reverse direction, where B10 cells were the responders, no MLR was observed (stimulation index of 0.9).

For comparison of MLR in bm25 and B10 cells, responses of mutant and standard cells to allogeneic BALB/c and B10.A cells that differ in the entire H-2 complex from the reactive cells are also shown in Fig. 1.

DISCUSSION

A number of mutations have been described in the mouse major histocompatibility complex H-2 (Klein et al., 1983; Mouse News Lett., H-2 Bulletin 1983, 1986, 1989). From a biochemical analysis performed in products of H-2 mutations it ensued that those mutations are involved which are manifested at the level of the H-2 molecule by substitution of one to several amino acids in the protein chain (Nairn et al., 1980; Klein et al., 1983). On the basis of nucleotide sequences of some H-2 mutations and comparison of them with nucleotide sequences of H-2 genes of other H-2 haplotypes, gene conversion has been suggested as a probable mechanism of the generation of H-2 mutations (Weiss et al., 1983).

Mutational events occurred in various H-2 haplotypes, but a mutation in the H-2^b haplotype has been the most frequent (Melvold and Kohn, 1975; Klein et al., 1983). At present 28 mutations in the b haplotype are known, they are designated bml to bm25, and most of them have been mapped in the K^b region. The K^b region of MHC thus represents a site with the highest mutation frequency (Klein, 1978).

H-2 mutations are most frequently detected by skin graft rejection. Apart from histogenetic changes they show a positive MLR, graft-versus-host reactivity and cell-mediated lympholysis between the mutant and parental strain, and serological changes in their phenotype. Serological studies of H-2 mutants demonstrated changes in known H-2 antigens (Klein et al., 1974; Apt et al., 1975; Mnatsakanyan et al., 1977) that, in some cases, were asso-

ciated with a definition of new and a loss of known H-2 specificities (Dishkant et al., 1973; Klein et al., 1975; David et al., 1977; McKenzie et al., 1977; Morgan et al., 1980b). In serological studies an examination of H-2^b mutations was the most difficult. Using defined H-2 antisera against the known H-2 specificities, some investigators failed to confirm the described serological differences between the mutant and parental strain (McKenzie et al., 1976), or the differences they detected were quantitative only (Klein et al., 1974).

Attempts to prepare antibodies by reciprocal immunization between parental and H-2^b mutant strains also often failed (Klein, 1978).

Mutation bm25 detected in transplantation tests performed in (B10.RIII × B10)Bc3 hybrids falls in the group of H-2^b mutations, and in serological analysis we made use of both usual approaches. Using a panel of well defined antibodies against known H-2 specificities, H-2^bbm25 cells were examined with an aim to determine whether any known H-2 antigen was altered, lost, or gained and, moreover, reciprocal immunization between the standard and bm25 mutant strain was carried out in order to obtain antibodies that would facilitate detection of any antigenic change.

Serological examination revealed some differences in cytotoxic reactivity of polyclonal H-2 antibodies to mutant and standard cells (lower titres of some anti-H-2^b antisera with bm25 cells), but the difference between bm25 cells and B10 cells was not evident until their reactivity to monoclonal antibodies K9 178 and HB 41 appeared. These monoclonal antibodies reacted only with standard cells and appeared more effective in detecting the difference between the mutant and standard strain than conventional H-2 antisera.

Monoclonal antibodies were also used with success in serological analysis of bm23, a product of another H-2^b mutation, and facilitated a serological distinction not only between standard and mutant cells but also between various types of mutant cells (Egorov and Egorov, 1984). Because the specificity of monoclonal antibody HB 41 was exactly known (anti-H-2.m39), we could conclude that the mutational change in the H-2 molecule occurred in bm25 cells at a site which corresponds to the specificity H-2.39. In subsequent absorption tests we supposed that bm25 cells would not remove the anti-H-2.39 antibody activity from antiserum ASP 235 and antibody HB 41 and that the absorbed antibodies would retain their capacity to react to H-2.39 positive strains. Contrary to our expectation, the bm25 cells retained some absorption ability and did absorb the anti-H-2.39 antibodies. The absorption results, nevertheless, suggest that expression of the specificity H-2.39 on bm25 cells is diminished.

In its properties, i.e. either weaker or no reactivity to antibodies in the direct cytotoxicity tests, while retaining some absorption ability, the bm25 mutation resembles other H-2 mutations, such as H-2^bm10 (Morgan et al., 1980a) and H-2^a (Klein et al., 1976).

In studying the bm25 mutation we also carried out reciprocal immunization between B10.RIII-H-2^{bm25} and the B10 strain, or its F₁ hybrids, but no antibodies were obtained. It is very difficult to prepare antibodies by immunization with cells having a small antigenic difference between donor and recipient, and our negative results are in agreement with the negative experiences in preparation of antibodies by reciprocal immunization between B10, or B6 strain, and other H-2^b mutants (Klein, 1978; Morgan et al., 1980a, c).

To complete a characterization of the bm25 mutation, bm25 cells were tested against B10 cells in mixed lymphocyte culture. A positive MLR was noted in the mutant—standard strain combination where B10.RIII-H-2^{bm25} cells were the responders. In the opposite direction, where B10 cells were the responders, no MLR was observed. If mutations are considered with respect to their phenotypic manifestations as mutations lost, gained, or lost and gained at the same time, the MLR results point to a loss of bm25 mutation. The results of reciprocal transplantation between B10.RIII-H-2^{bm25} and B10 cells, where rejection occurred in both directions (Blandova and Ratkin, 1987), as well as the serological analysis of bm25 cells demonstrate however that the bm25 mutation is not only a lost mutation.

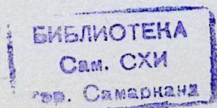
The results of serological analysis and MLR in cells carrying the bm25 mutation can be briefly summarized:

- bm25 cells are serologically demonstrable in the direct cytotoxicity test with monoclonal antibodies HB 41 and K9 178. In this case, monoclonal antibodies appeared to be a finer tool for the detection of H-2 mutation than conventional H-2 antisera.
- Monoclonal antibody HB 41 directed against the specificity H-2.39 allowed a precise determination of what part of the H-2 molecule was altered by a mutation: the mutation changed the epitope which represents the specificity H-2.39.
- Serological tests with HB 41 antibody confirmed indirectly the location of bm25 mutation in the H-2 complex: specificity H-2.39 is mapped in the H-2K^b region and this H-2 specificity was altered in mutant cells.
- A positive MLR in the mutant—standard strain combination was observed only when B10 cells were the stimulators.

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Серологический анализ и смешанная лимфоцитарная реакция (MLR) клеток, несущих спонтанную мутацию H-2^{b_m25}

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Резюме. Серологический анализ мутантных клеток H-2^{b_m25} обнаружил, что мутация охватила в области K^b комплекса H-2 то место, которое отвечает специальности H-2,39. Моноклональное антитело анти-H-2.m39 НВ 41 не реагировало в прямом цитотоксическом тесте с мутантными клетками b_m²⁵, тогда как реакция со стандартными клетками В10 оставалась неизменной. Однако клетки В10.R111-H-2^{b_m25} сохраняли определенную способность к абсорбции и в тестах абсорбции были способны отчасти удалять антитело анти-H-2.39. В смешанной лимфоцитарной реакции клетки b_m²⁵ отвечали пролиферацией на стимулирующие стандартные клетки В10, но в противоположном случае положительная MLR не была обнаружена.

Enhancement of Haemopoietic Recovery in Sublethally Gamma-irradiated Mice by the Joint Use of Indomethacin and Cystamine

(gamma-radiation / recovery of haemopoiesis / cystamine / indomethacin)

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Received April 24, 1990

Abstract. The effects of indomethacin and cystamine, administered prior to gamma-irradiation of mice, were investigated. Synergistic enhancement of haemopoietic recovery was observed under the conditions of a joint use of both drugs. The reason for this synergistic action is probably a joint operation of two mechanisms, i.e., the lowering of the radiosensitivity of haemopoietic stem cells by cystamine and the increase of the proliferation of surviving haemopoietic cells by indomethacin.

The combined use of means effective in reducing radiation damage of the organism by different mechanisms of action belongs to current trends in radioprotection (Walker, 1988). In the light of the leading role of haemopoietic depression in the pathogenesis of the acute radiation syndrome, attention is focused on the combination of agents which decrease the radiosensitivity of haemopoietic stem cells, and of means which enhance the proliferation and differentiation of the surviving stem cells. If agents are administered that contribute to stem cell survival (like sulfhydryl compounds protecting by scavenging free radicals), followed or preceded by agents that enhance stem cell repopulation, then even more dramatic effects in radioprotection may be obtained. Stem cell repopulation can be modified by intervention into the regulatory pathways of the haemopoietic cell renewal populations. The physiological control of haemopoiesis involves complex interactions between stimulatory and suppressive endogenous molecules. One regulatory biomolecule which participates in the control of the proliferation of haemopoietic progenitor cells is prostaglandin E_2 . It was shown that prostaglandin E_2 inhibited the proliferation of granulocyte-macrophage progenitor cells in mice (Kurland and Moore, 1977; Gentile et al., 1983). It was hypothesized that some types of prostaglandins might play the role of inhibitory factors in the regulation of haemopoiesis, and attempts were made to use non-steroidal anti-inflammatory drugs which suppress the prostaglandin generation via cyclo-oxygenase inhibition, as pharmacological means of positive haemopoietic control. Fontagné et al. (1980) have shown that a decrease of prostaglandin levels in bone marrow cells of indomethacin-treated mice is correlated with the rise in the recognizable myeloid precursors. Our experiments using sublethally gamma-irradiated mice injected post-radiation with indomethacin have demonstrated stimulatory effects of this treatment on bone marrow and spleen cellularity, peripheral leucocyte

counts and cell proliferation in haemopoietic organs (Pospíšil et al., 1986). In our further experiments a stimulatory effect of indomethacin on the proliferation of haemopoietic stem cells of bone marrow grafts was observed in lethally irradiated mice (Kozubík et al., 1987). Even a single-dose pre-irradiation administration of non-steroidal anti-inflammatory drug representatives, i.e., indomethacin and diclofenac, was found to be effective in enhancing haemopoietic recovery in the spleens of gamma-irradiated mice (Kozubík et al., 1989).

The purpose of the present experiments is to explore the possibility of a joint pre-irradiation administration of cystamine, a radioprotector acting via decrease of radiosensitivity, and indomethacin with the expected potency to enhance post-radiation recovery. The results suggest favourable effects of the joint operation of both drugs.

MATERIALS AND METHODS

Animals

Conventional male mice (CBA/JPh $\times$ C57BL/10CsSnPh)F₁ were kept under controlled lighting (LD 12 : 12) at a temperature of $22 \pm 2^\circ\text{C}$; 20 animals per cage. Pelleted standard laboratory diet (DOS-2b-St Velaz) and HCl-treated tap water (pH 2–3) were given ad libitum. At the age of 12–14 weeks (30 ± 3 g body weight) the mice were subjected to the experimental procedure. Control and experimental procedures were carried out concurrently in mice from the same cage and the groups were pooled.

Irradiation

The mice were irradiated with single sublethal whole-body doses from a ^{60}Co gamma-ray source (Chisostat, Chirana) at a dose rate of 0.4 Gy/min. During irradiation the mice were placed individually in chambers in a slowly rotating circular perspex container.

Administration of drugs

Indomethacin (Sigma) was dissolved in ethanol (10 mg/ml), diluted in an isotonic phosphate buffer (pH 7.4) and injected i.p. in amounts of 0.15 mg per mouse, in volumes of 0.2 ml, 60 min before irradiation. Cystamine dihydrochloride was administered in saline i.p. in amounts of 3 mg per mouse, in volumes of 0.2 ml, 10–15 min before irradiation. Control animals were i.p. injected with the same volumes of solutions used for dilution of the drugs.

Determination of haemopoietic indices

Peripheral leucocytes and nucleated elements of femoral bone marrow were estimated by means of a Coulter Counter. Blood samples were drawn from a

fine incision in the tail vein. The bone marrow of femoral diaphyses was washed with saline and suspended. Wet weights of the spleens were determined at the selected postradiation intervals. On day 10 after irradiation the recording of macroscopic endogenous spleen colonies counted on the parietal side of the spleen fixed in Bouin's solution was performed.

The assay of cell proliferation (Hughes et al., 1964) consisted of ^{125}I -iododeoxyuridine incorporation into the DNA of the spleen cells. The mice were given i.p. 3.7×10^4 Bq ^{125}I -iododeoxyuridine (Institute of Nuclear Research, Praha) in 0.4 ml saline. In order to inhibit endogenous thymidylate synthesis, 10^{-7} mol 5-fluoro-2'-deoxyuridine (Serva, FRG) was injected i.p. 30 min before ^{125}I -iododeoxyuridine. After 6 h the mice were killed by cervical dislocation and the spleens were placed in 10% buffered formalin for 48 h to remove radioactive iodine not incorporated into DNA. The radioactivity of the organs was measured with a well-type counting system and expressed as percentage of the activity administered.

Statistics

The values given in the figures and the table represent the means $\pm$ SEM. The statistical significance of the differences was evaluated using Student's *t*-test. The regression lines for the endogenous spleen colony numbers were calculated by the least-squares method. For each point at least 8 animals were used.

RESULTS

The cellularity of femoral bone marrow, the leucocyte counts in peripheral blood and spleen weights were evaluated at various intervals after irradiation of the animals with 6.5 Gy in control mice, mice protected with indomethacin or cystamine alone and an indomethacin—cystamine combination (Figs. 1–3). A typical postirradiation course of the measured indices, showing a dip, recovery and attaining the normal levels in the 4th postirradiation week, is evident. With respect to nucleated cells in the femur (Fig. 1), indomethacin alone has little effect, evident potentiation of the recovery is obtained with cystamine alone, the highest rate of recovery is achieved using the indomethacin—cystamine combination. Postirradiation blood leucocyte counts (Fig. 2) indicate a similar degree of elevation when indomethacin or cystamine are given alone, the highest rate of recovery being again observed in mice protected with the combination of indomethacin and cystamine. The simultaneously determined red blood cell counts did not exhibit any significant differences between control and experimental groups (data not shown). As demonstrated in Fig. 3, indomethacin or cystamine given alone modify only slightly the early recovery phase of splenic weight; the administration of the indomethacin—cystamine combination leads to a profound enhancement of

the organ weight recovery. It is worth noting that the overshoot of spleen weight, occurring by the end of the 3rd postirradiation week in control mice, is damped by the protective agents used.

Fig. 4 gives the numbers of endogenous spleen colonies calculated as a function of the radiation dose on day 10 after irradiation in control mice and mice protected by the agents tested. As shown by the character of the cal-

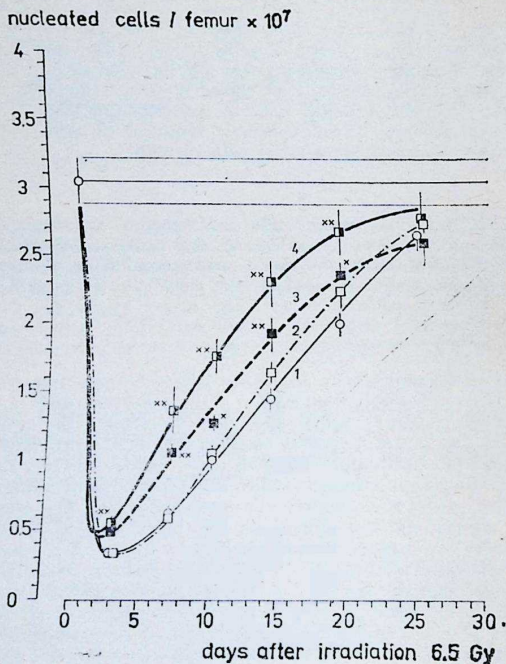


Fig. 1. Changes in the number of nucleated cells of the femur in control (1) and experimental animals treated with indomethacin (2), cystamine (3) and indomethacin-cystamine combination (4) monitored before ("0") and at various intervals after irradiation with 6.5 Gy. Difference from controls: x $P < 0.05$, xx $P < 0.01$.

culated regression lines, indomethacin alone elevates the colony numbers without modifying the slope of the lines, the administration of cystamine alone decreases the slope of the dose-response dependence, the joint use of indomethacin and cystamine leads to a combination of both effects and to the highest degree of colony formation. The evidence of the high stimulation of cell proliferation processes in spleens of mice protected with the indomethacin—cystamine combination are results of measurement of ^{125}I -iodo-

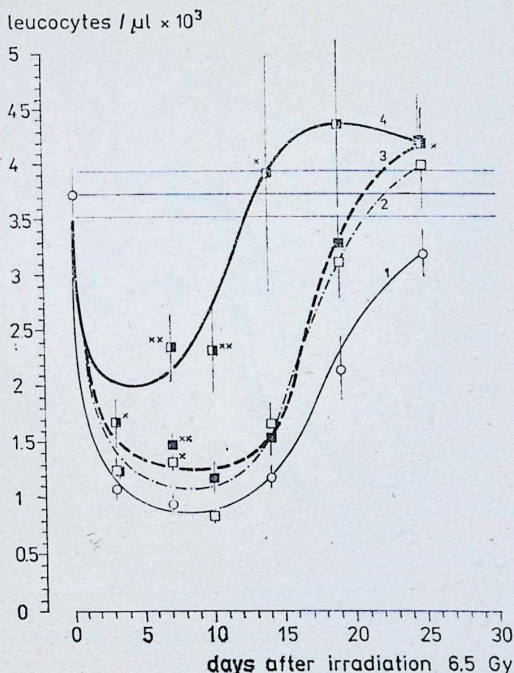


Fig. 2. Numbers of leucocytes in control (1) and experimental animals treated with indomethacin (2), cystamine (3) and indomethacin-cystamine combination (4) measured before ("0") and at various intervals after irradiation with 6.5 Gy. Difference from controls: x $P < 0.05$, xx $P < 0.01$.

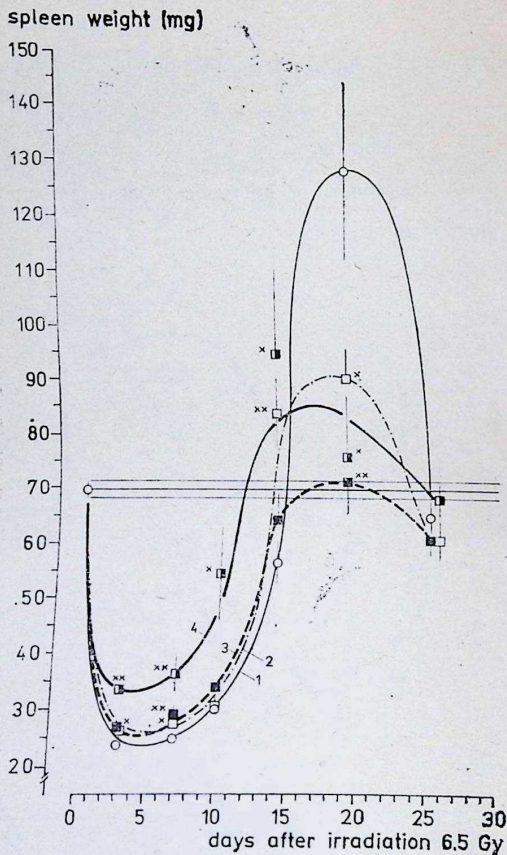


Fig. 3. Changes in the spleen weight in control (1) and experimental animals treated with indomethacin (2), cystamine (3) and indomethacin-cystamine combination (4) monitored before ("0") and at various intervals after irradiation with 6.5 Gy. Difference from controls: x $P < 0.05$, xx $P < 0.01$.

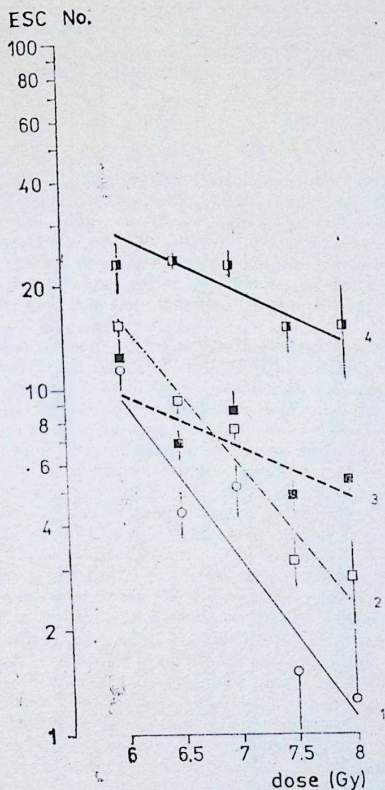


Fig. 4. Regression lines for the dependence of endogenous spleen colony counts (ESC) on radiation dose (6–8 Gy) in control (1) and experimental animals treated with indomethacin (2), cystamine (3) and indomethacin–cystamine combination (4). Differences in ESC counts in mice irradiated with 7.5 Gy ($n = 16-20$) are statistically significant at the 0.01 level when comparing groups 1VS2, 1VS3, 1VS4, 2VS4, 3VS4.

Table 1. Percentage of ^{125}I -iododeoxyuridine uptake in spleens on day 10 after irradiation

Exp. group	Dose (Gy)			
	6.5	7.0	7.5	8.0
Indomethacin + cystamine	2.13 ± 0.56 $P < 0.001$	0.87 ± 0.12 $P < 0.01$	0.33 ± 0.05 $P < 0.01$	0.34 ± 0.11 $P < 0.01$
Cystamine	0.26 ± 0.06 $P < 0.01$	0.12 ± 0.01 $P < 0.05$	0.10 ± 0.03 NS	0.08 ± 0.02 NS
Indomethacin	0.22 ± 0.04 $P < 0.05$	0.08 ± 0.02 NS	0.08 ± 0.01 NS	0.06 ± 0.01 NS
Controls	0.13 ± 0.02	0.07 ± 0.01	0.06 ± 0.02	0.05 ± 0.01

P — statistical significance as compared to controls (NS — statistically non-significant).

deoxyuridine uptake in spleens (Table 1). While indomethacin or cystamine alone significantly increases the uptake values only at smaller radiation doses used, the protection with the indomethacin—cystamine combination strongly elevates this indicator of cell proliferation at all the radiation doses used.

DISCUSSION

The results suggest that the pre-irradiation administration of indomethacin and/or cystamine enhances the postirradiation recovery of the haemopoietic indices measured, the joint use of both drugs giving the highest effect. As shown in the case of the peripheral leucocyte counts, spleen weight and cell proliferation in the spleen, the joint use of indomethacin and cystamine gives effects greater than additive, for which the term synergism is used. The reason of the synergistic enhancement of the recovery seems to be different mechanisms of action of both drugs.

Cystamine, a well-known and potent radioprotector acting via mechanisms such as free-radical scavenging and hypoxia (Bacq, 1965), decreases the radiosensitivity of haemopoietic stem cells and induces their higher postirradiation survival. The reflection of this mechanism might be the decreased slope of the dose-response curves of endogenous spleen colonies, shown in our experiments using cystamine administration and described by other authors when evaluating the effects of radioprotective drugs on haemopoietic stem cells (Smith et al., 1966; Phillips and Hanks, 1968). Indomethacin does not modify the radiosensitivity of spleen colony-forming units. As shown in our experiments, indomethacin treatment displaces only the survival curve of endogenous colony-forming units by about 0.75 Gy to the right of the curve of controls. As discussed by Smith and McKinley (1976), such a situation means either an increase in the number of haemopoietic stem cells at risk at the time of irradiation, or an increase in the postirradiation proliferation of stem cells. Due to the short interval between indomethacin administration and radiation exposure, the latter possibility is probable. The indomethacin action stimulating cell proliferation can operate in the postirradiation period and enhance the proliferation of stem cells, which in normal conditions starts during the first day after irradiation (Chervenick and Boggs, 1971). If such a mechanism is in play together with the increased survival of haemopoietic stem cells, a high and effective amplification of the repopulation potential of stem cells can be expected. Increased size of the colony-forming cell population is reflected in their progeny and leads to earlier restoration of the counts of mature blood cells in the later postirradiation period.

The mechanism of the stimulatory indomethacin action was discussed already in the introduction. It was deduced that the influence of indomethacin on haemopoiesis could be explained by its inhibitory action on prostaglandin

generation and thus by the removal of the negative effects of endogenous prostaglandins on the proliferation of haemopoietic cells (Kurland and Moore, 1977; Fontagné et al., 1980; Gentile et al., 1983).

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Усиление восстановления кроветворения у мышей после сублетального гамма облучения совместным применением индометацина и цистамина

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Резюме. Исследовались эффекты индометацина и цистамина, поданных мышам перед гамма облучением. При условии совместного применения обеих веществ наблюдалось синергическое усиление восстановления кроветворения. Причиной этого синергического действия является по всей вероятности совместное действие двух механизмов: т.е. снижение радиочувствительности стволовых клеток кроветворения в результате применения цистамина и повышение пролиферации выживших кроветворных клеток в результате действия индометацина.

Active Suppression of the Proliferative Response to Interleukin-2 in Tumour-bearing Mice

(IL-1 / IL-2 / cell proliferation / indomethacin / suppressor cells)

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Received May 14, 1990

Abstract. Spleen cells from mice bearing progressively growing syngeneic methylcholanthrene-induced sarcomas are immunologically hyporeactive and also their proliferative responses to interleukin-1 (IL-1) or interleukin-2 (IL-2) stimulation are considerably decreased. The hyporeactivity to IL-2, but not to IL-1, is due to an active suppression: spleen cells from tumour-bearing mice inhibit the IL-2-induced proliferation of cells from normal donors. Supernatants obtained after cultivation of spleen cells from mice bearing tumours show similar suppressive effects. The hyporeactivity to IL-2 and IL-1 in tumour-bearers is not improved by indomethacin, an inhibitor of prostaglandin synthesis. The results show that the low reactivity to IL-2 in tumour-bearing mice is due to an active suppression mediated by spleen cells and their factor(s) and that more different mechanisms regulate responsiveness to interleukins in tumour-bearing hosts.

Immunotherapy by means of cytokines has emerged as a promising approach to treatment of cancers. Encouraging results, especially with interleukin-2 (IL-2), have been obtained in both animal (Bubeník et al., 1983; Rosenberg et al., 1985; Lafreniere and Rosenberg, 1985; Thompson et al., 1986) and human (Rosenberg, 1984; Mulé et al., 1985; Rosenberg et al., 1987; West et al., 1987) systems. However, the success of such treatment depends on a number of variables, such as tumour size, type and localization of tumour, etc. (Lotze et al., 1986; Oliver, 1988). In general, the efficacy of the treatment diminished with increment of tumour burden, and this correlates with a decrease of immunological reactivity observed in bearers of progressively growing tumours (Young et al., 1973; Howell et al., 1975; North, 1985).

We have shown previously that spleen cells from immunologically hyporeactive mice bearing progressively growing syngeneic sarcomas had also decreased proliferative responses to exogenous interleukin-1 (IL-1) and IL-2 (Holáň and Lipoldová, 1990a). This hyporeactivity was associated with impaired ability to synthesize functional high-affinity IL-2 receptors (Holáň and Lipoldová, 1990a,b). Here we show that the low proliferative response to IL-2 in spleen cells from tumour-bearing mice is due to an active suppression that is not mediated by prostaglandins. Hyporeactivity to IL-1 seems, on the contrary, to be a property of the responsive cells themselves. The results thus show that different mechanisms regulate the responsiveness to interleukins in tumour-bearing hosts.

Abbreviations: IL-1 — interleukin-1, IL-2 — interleukin-2, IL-2R — receptor(s) for IL-2.

MATERIALS AND METHODS

Animals

Male mice of the inbred strain C57BL/10SnSc (hereinafter B10), 2–3 months of age, were used in all experiments. Mice were from the breeding colony of our Institute.

Tumours

Sarcoma MC 11 originally induced by methylcholanthrene in a B10 male (Bubeník et al., 1978) was used. To induce tumours, 10^5 MC 11 cells in a volume of 0.1 ml of phosphate-buffered saline were injected subcutaneously into mice. Spleen cells from tumour-bearing mice were tested when the tumours reached a size approximately 2 cm^2 (roughly 3 weeks after tumour cell inoculation).

Interleukins

Recombinant human interleukin-1 α (rIL-1 α , specific activity 1×10^8 units/mg) was obtained from Dr. P. T. Lomedico, Hoffman-La Roche Inc., Nutley, N. J. (Gubler et al., 1986). The activity of rIL-1 was tested in the classical thymocyte co-stimulatory assay (Mizel et al., 1978). Since the activity of IL-1 changes during the storage and manipulation with it, all samples were retested before using. The number of units for each IL-1 sample was taken as the reciprocal of the IL-1 dilution that gave half the maximum [^3H] thymidine incorporation, as was described in detail by Otterness et al. (1988).

Recombinant human interleukin-2 (rIL-2, specific activity 2×10^6 units/mg) was obtained from Dr. E. Gren, Institute of Organic Synthesis, Riga, USSR. The activity of rIL-2 was assessed by its ability to support growth of IL-2-dependent CTTL (Gillis and Smith, 1977), and one unit of IL-2 is defined as the highest dilution of IL-2 preparation giving half the maximum [^3H] thymidine incorporation in CTTL.

Preparation of supernatants

Spleen cells from control and tumour-bearing mice were cultured at a concentration of 5×10^6 cells/ml in Eagle's minimal essential medium supplemented with antibiotics (penicillin, streptomycin), glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated bovine serum (complete Eagle's medium). After 24-h cultivation at 37°C in an atmosphere of 5% CO_2 in air, the cultures were centrifuged and the supernatants were aliquoted and stored at -25°C until used.

Proliferative assay

Spleen cells from control and tumour-bearing mice were cultured at a concentration of 1.8×10^5 cells/well in 96-well flat-bottomed plates (Sterilin

Ltd., Feltham, England) in 0.2 ml of complete Eagle's medium. Recombinant IL-1 or IL-2 was added to the cultures to make final concentrations 100 units/ml. Indomethacin (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 10^{-5} M was added to some cultures. The plates were incubated at 37°C and [3 H]thymidine (1 μ Ci/ml) was added to the cultures for the last 6 h of the 72-h incubation period. Cells were harvested onto glass fibre filters and the activity incorporated was determined by liquid scintillation. The results are expressed as counts per min (cpm) $\pm$ standard deviation.

Suppressor cell activity assay

Spleen cells (1.2×10^5 /well) from control mice were stimulated alone or in the presence of cells from tumour-bearing mice (at ratios 1 : 1 to 32 : 1). To test the activity of supernatants, the samples under study were added to the wells containing spleen cells (1.8×10^5 cells/well) from control mice to make a final dilution of supernatants 1 : 4 to 1:128. Recombinant IL-1 or rIL-2 (100 units/ml) was added to the cultures and the proliferation of cells was determined by adding [3 H]thymidine (1 μ Ci/ml) for the last 6 h of the 72-h incubation period.

Statistical analysis

The statistical significance of the reactions was estimated by Student's *t*-test.

RESULTS

Spleen cells from mice bearing progressively growing methylcholanthrene-induced sarcomas are immunologically hyporeactive and their proliferative responses to IL-1 and IL-2 stimulation are significantly ($P < 0.001$) decreased (Fig. 1).

We tested whether the low reactivity of spleen cells from tumour-bearing mice to interleukins is due to an active suppression. Interleukin-2-induced proliferation in a mixture of spleen cells from tumour-bearing and control animals was compared with proliferation of control cells alone. As shown in Fig. 2, spleen cells from tumour-bearing mice inhibit, in a dose-dependent manner, proliferation of cells from control donors. This suppression was not due to an overcrowding of the wells with cells, since twice more cells from normal mice, than were used, displayed a higher degree of proliferation than the number of normal cells used in mixture experiments (data not shown).

The suppression of IL-2-induced cell proliferation, which was observed in a mixture of cells from control and tumour-bearing animals, was not found

when the cultures were stimulated with IL-1. The proliferation in IL-1-stimulated cultures of cells from control and tumour-bearing mice was always higher than that observed in cultures containing only cells from control animals (Fig. 3).

The low proliferation in cultures of spleen cells from tumour-bearing mice and also suppression of IL-2-induced proliferation could be due to a suppressive action of prostaglandins produced by tumour-host derived macrophages. We therefore next tested proliferation of spleen cells from tumour-bearing mice, which were stimulated with IL-2 in the presence of indomethacin. Indomethacin only slightly increased IL-2-induced proliferation of

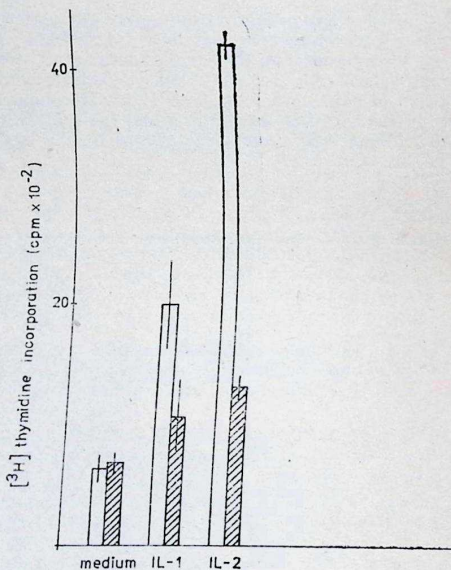


Fig. 1. Hyporeactivity to IL-1 and IL-2 stimulation of spleen cells from tumour-bearing mice. Spleen cells from control (open columns) or tumour-bearing (hatched columns) B10 mice were cultured unstimulated or were stimulated with 100 units/ml of IL-1 or IL-2.

spleen cells from both normal and tumour-bearing mice, but the low responsiveness of cells from tumour-bearing hosts was not reversed (Fig. 4).

Finally, we have tried to determine, whether the suppression of IL-2-induced cell proliferation requires cell-to-cell contact or whether it is mediated by cell-free molecules. Spleen cells from control and tumour-bearing mice had been cultured for 24 h alone and the supernatants obtained were tested for the ability to inhibit IL-2-induced proliferation of spleen cells from normal mice. Results shown in Fig. 5 demonstrate that supernatants obtained after cultivation of spleen cells from tumour-bearing mice inhibited cell proliferation to a significantly higher degree than did control supernatants.

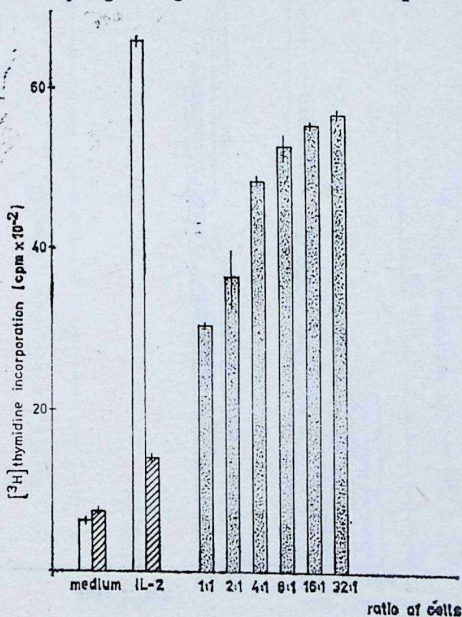


Fig. 2. Spleen cells from tumour-bearing mice inhibit responsiveness of cells from control mice to IL-2. Spleen cells from tumour-bearing (hatched columns) or control (open columns) B10 mice were unstimulated or were stimulated with IL-2 (100 units/ml) alone or the mixture of both types of cells was stimulated with IL-2 (stippled columns).

DISCUSSION

The phenomenon of depression of immunological reactivity in bearers of progressively growing syngeneic tumours is well established (Young et al., 1973; North, 1985). Different mechanisms have been considered for this depression, and the suppressor cells have been demonstrated as the most important among them (Howell et al., 1975; Veit and Feldman, 1976; Berczi

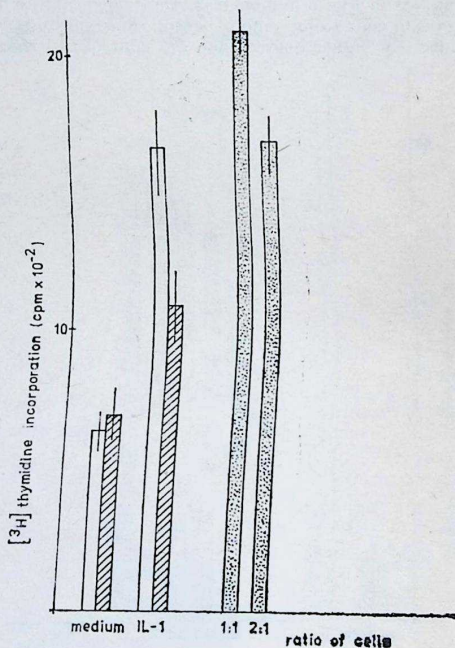


Fig. 3. Interleukin-1-induced proliferation of spleen cells from control mice is not inhibited by spleen cells from tumour-bearing mice. Spleen cells from tumour bearing (hatched columns) or control (open columns) B10 mice were stimulated with IL-1 (100 units/ml) alone or the mixture of both type of cells was stimulated with IL-1 (stippled columns). The ratio of cells from control to tumour-bearing mice was 1 : 1 and 2 : 1.

and Sehon, 1979; Hellström and Hellström, 1981). These tumour-induced suppressor cells do not inhibit only immune responses to tumour antigens, but also to unrelated antigens or mitogens.

We have described a decrease of proliferative responses to IL-1 and IL-2 in mice bearing progressively growing syngeneic sarcomas (Holáň and Lipoldová, 1990a), and Kradin et al. (1989) also observed reduced proliferative

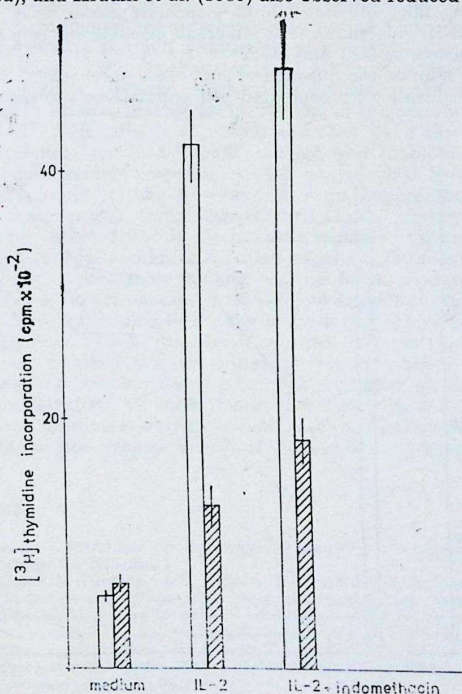


Fig. 4. Effect of indomethacin on IL-2-induced proliferation of spleen cells from tumour-bearing mice. Spleen cells from control (open columns) or tumour-bearing (hatched columns) B10 mice were cultured unstimulated or were stimulated with IL-2 (100 units/ml) alone or with IL-2 in the presence of 10^{-5} M indomethacin.

responses to IL-2 in cancer patients. We tested whether this hyporeactivity to stimulation with interleukins is due to an active suppression or whether it is a result of changes in responding cells from tumour-bearing hosts. The results showed that the low responsiveness of tumour-bearing mice to IL-2 is due to an active suppression, similarly as is regulated the hyporeactivity to mitogens (Veit and Feldman, 1976). On the contrary, spleen cells from tumour-bearing mice did not inhibit responsiveness of cells from control animals to IL-1. This shows that different mechanisms can regulate proliferative responses to IL-1 and IL-2.

We did not address the question of suppressor mechanism. Since our preliminary experiments with separated cell populations showed that the ob-

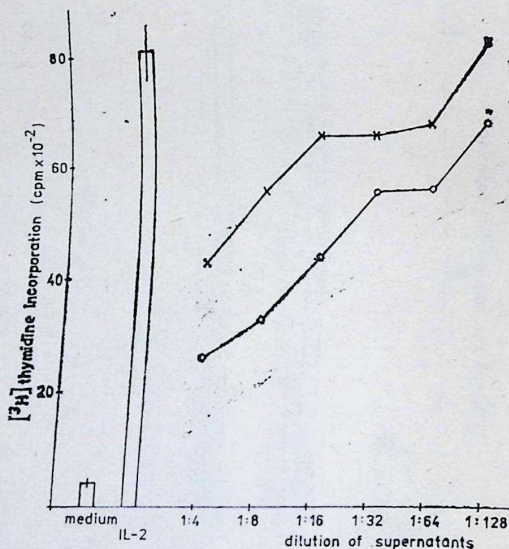


Fig. 5. Effect of supernatants obtained after cultivation of spleen cells from control (x—x) and tumour-bearing (O—O) mice. Supernatants obtained after 24-h incubation of spleen cells were tested for the ability to inhibit IL-2-induced proliferation of spleen cells from control B10 mice.

served low reactivity to IL-2 is retained also after removing macrophages from hyporeactive cells of tumour-bearers (data not shown), the macrophages do not seem to be an inhibitory cell population. This is supported by the observation that the hyporeactivity of tumour-bearer's cells to IL-2 is not reversed by indomethacin, an inhibitor of prostaglandins produced predominantly by macrophages. In systems of suppression of immunological responses to mitogens or antigens in tumour-bearing hosts it was demonstrated that macrophages/monocytes could be an inhibitory cell population mediating suppression through production of prostaglandins (Pope et al., 1976; Nakata et al., 1988; Parhar and Lala, 1988; Chao and Chu, 1989).

There appear to be different mechanisms regulating the responsiveness to interleukins in tumour-bearing hosts. Bearers of tumour show diminished production of IL-2 (Kradin et al., 1989) and they have also impaired expression of receptors for IL-2 (IL-2R) (Holář and Lipoldová, 1990b). In addition, the proliferative response to IL-2 is controlled by a suppressive mechanism. It is not known whether the same suppressive mechanism, which inhibits cell proliferation, also inhibits IL-2 production and IL-2R expression, or whether different mechanisms regulate synthesis of and response to IL-2. A quite another mechanism regulates the reactivity to IL-1. Immunologically hyporeactive tumour-bearing hosts produce comparable amounts of IL-1 as do normal donors (Holář and Lipoldová, 1990c), but their reactivity to IL-1 is diminished. This decrease is not controlled by the suppressive mechanism, as is in the case of hyporeactivity to IL-2.

Altogether the results demonstrate that the responsibility to IL-1 and IL-2 of spleen cells from tumour-bearing hosts is regulated on different levels. In a final manifestation, all these immunoregulatory mechanisms lead to a decrease of proliferative reactivity to interleukins, similarly as is decreased general immunological responsiveness of tumour-bearing hosts.

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Активное подавление пролиферативной реакции на интерлейкин 2 у мышей-носителей опухоли

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Резюме. Клетки селезенки от мышей-носителей прогрессивно растущей сингенной саркомы, вызванной метилхолантеном, являются иммунологически гипореактивными, и их пролиферационная реакция на стимуляцию интерлейкином-1 (IL-1) или интерлейкином-2 (IL-2) значительно понижена. Гипореактивность к IL-2, но не к IL-1, вызывается активным подавлением: клетки селезенки от мышей, несущих опухоль, угнетают вызываемую с помощью IL-2 пролиферацию клеток от нормальных доноров. Надосадочная жидкость, полученная путем культивации клеток селезенки от мышей с опухолью, обнаруживает аналогичное угнетающее действие. Гипореактивность к IL-1 и IL-2 у носителей опухолей, не повышается под действием индометацина, угнетающего синтез простагландинов. Эти результаты показывают, что низкая реактивность к IL-2 у мышей, несущих опухоль, вызывается активным подавлением, опосредованным клетками селезенки и их фактором (факторами), и что реакцию на интерлейкины у носителей опухолей регулирует несколько разных механизмов.

Microtubular Dynamics of *Saccharomyces cerevisiae* Temperature-sensitive Secretory Mutants: The *sec 1* Product and Polymerization of Microtubules (

(secretion / yeast / cytoskeleton)

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Received February 12, 1990

Abstract. The relation of cytoplasmic microtubules to intracellular transport was studied in temperature-sensitive (ts) secretory mutants of *Saccharomyces cerevisiae* at permissive and nonpermissive temperature using indirect immunofluorescence with monoclonal antibody TU-01 against α -tubulin. It was found that in the *sec 1* mutant, which at 37°C accumulated secretory vesicles and in which therefore transport of secretory material from secretory vesicles to cytoplasmic membrane was inhibited, cytoplasmic and in some cases nuclear microtubules were impaired. After 4 h of postcultivation at 24°C the altered phenotype reverted to the original state, the cells began to divide, and were comparable with control. Use of the *sec 1* mutant protoplasts suggested that the product, whose gene is mutated, is probably involved in microtubular polymerization. The *sec 7* mutant, which accumulates the Golgi complex under nonpermissive conditions and in which the transfer of secretory material from the Golgi complex to the secretory vesicles is thus inhibited, showed no significant changes in the length or number of cytoplasmic microtubules. As a result of secretory product accumulation, the cytoplasmic microtubules were displaced towards the periphery in some cells.

A secretory process in a cell consists of several steps: synthesis of the particular protein, its translocation and posttranslational modification in the endoplasmic reticulum (ER), its transport from ER to the Golgi complex (GC) and hence to the secretory vesicles (SV), and the exocytosis itself. A number of the ts secretory mutants of *Saccharomyces cerevisiae* have been isolated (Novick and Schekman, 1979; Novick et al., 1980) that have those genes defective whose products are involved in secretion. According to their phenotype under nonpermissive conditions, they have been divided into mutants accumulating ER, GC, or SV. Each of these groups comprises several strains.

Parallel with the biochemical and genetical analysis of secretion (Ferro-Novick et al., 1984; Bernstein et al., 1985; Deshaies and Schekman, 1987; Newman and Ferro-Novick, 1987; Böhni et al., 1988; Nakano et al., 1988; Schekman et al., 1988) proceeds the study of the dynamics of cytoplasmic microtubules (cMTs) as one of the cytoskeletal components and their role in the transfer of SV through the cytoplasm. Their involvement in the secretory process has been reported in some cell systems: impairment of MTs by inhibitors results in deficient intracellular SV transport (Ericson, 1980; Cho and Garant, 1981; Busson-Mabillot et al., 1982; Bennet et al., 1984a, b).

Abbreviations: cMTs — cytoplasmic microtubules, nMTs — nuclear microtubules, SPB — spindle pole body, SV — secretory vesicles, ER — endoplasmic reticulum, GC — Golgi complex.

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Direct evidence of contact between cMTs and the material being transported has been furnished by methods of electron microscopy (Langford et al., 1987; Tooze and Burke, 1987) and videoenhanced DIC microscopy (Pryer et al., 1986). On the other hand, the involvement of cMTs in secretion has not been confirmed in some other cell systems (Rogalski et al., 1984; Virtanen and Vartio, 1986). The study of secretory related events has also been undertaken in *S. cerevisiae* (Huffaker et al., 1988; Jacobs et al., 1988). Two mutually independent investigations claim that in this yeast cMTs do not take part in the secretory process.

We made use of a different approach to the study of the cMTs—secretion relationship: the secretory process was inhibited in our model system and then the morphology and dynamics of MTs was analysed. The experiments were performed with two ts secretory mutants: *sec 1*, which accumulates SV, and *sec 7*, which accumulates GC at nonpermissive temperature of 37°C.

MATERIALS AND METHODS

Strains and cultivations

Saccharomyces cerevisiae wild type, haploid CCY 22-12-3, *Saccharomyces cerevisiae sec 1* HMSF 1 (*sec 1-1*), and *Saccharomyces cerevisiae sec 7* SF 294-2B (*sec 7-1*) originating from Dr. R. Schekman's laboratory were maintained on 2% wort agar at 4°C. Prior to the experiment proper, the *sec 1* and *sec 7* strains were cultivated on 2% wort agar at permissive (24°C) and nonpermissive (37°C) temperature. Only colonies that did not grow at 37°C were used in the study. They were transferred from the wort agar into liquid medium containing 0.3% yeast extract, 0.5% bactopectone, and 2% glucose and were agitated to early exponential phase. Growing cells were cultivated at 24° and 37°C for 1.5 h before fixation and treated for immunofluorescence. Part of the cells growing at 37°C was postcultivated for 2 or 4 h at 24°C for analysis of phenotype reversion. Another part of cells was used for generation of protoplasts: cells were washed twice in water and incubated in snail enzyme (20 mg enzyme/ml 0.6 M KCl) at 37°C for 1 h. Then, the protoplasts were washed twice in 0.6 M KCl and cultivated in osmotically stabilized medium for 1.5 h. A portion of protoplasts grew at 37°C and another at 24°C.

Indirect immunofluorescence

The treatment of samples was modified after Hašek et al. (1986), fixation was done in 3.5% formaldehyde in phosphate buffer (pH 6.5) containing 1 M KH_2PO_4 , 0.02 M MgCl_2 , and 0.01 M EGTA; two times washing in phosphate-buffered saline, extraction in methanol at -20°C for 10 min, then in acetone at -20°C for 1 min; preincubation in 2% bovine serum albumin for 20 min at 37°C, incubation 1 h in primary antibody TU-01 (Viklický et al., 1982)

at 37°C, three times washing in PBS, 45 min in secondary antibody (SWAM FITC SEVAC Praha) at 37°C, 3 × PBS, mounting in 0.1% p-phenylenediamine in 90% glycerol in PBS (pH 8.5).

Fluorescence microscopy

Observations were made and documentation provided with a Jenalumar Carl Zeiss fluorescence microscope (Jena) using epifluorescence and 530–560 nm filters, 1–2 min exposure, and a Fomapan 21 film.

RESULTS

Microtubules in wild-type *S. cerevisiae*

A temperature of 37°C represented the nonpermissive environment. The possibility had to be precluded that the temperature itself impaired cMTs in the wild-type. For this reason a haploid *S. cerevisiae* strain was used as control. Patterns of nuclear and cytoplasmic MTs typical for *S. cerevisiae* were found in the wild strain not only at permissive but also at 37°C. Nuclear MTs were seen during the S, the G₂ phase, and in mitosis; cMTs were the most developed at the G₁ phase. No substantial difference in MT morphology was found between permissive and nonpermissive temperature (Plate XLVI, Fig. 1 and 2).

Microtubules in *sec 1* mutant cells

Secretion of glycoproteins was arrested within 10–15 min of transfer of this strain to nonpermissive temperature; accumulation of SV began within 30 min (Nečas and Svoboda, 1986). At 24°C we observed typical arrangement of MT structures after 1.5 h. On the other hand, considerable reduction in MTs was evident in cells incubated at 37°C after the same interval. At the G₁, S, and G₂ phase most cells displayed only spindle pole bodies (SPBs) as bright points or SPBs connected with short nMTs. We cannot observe typical M phase because the cell cycle is not finished in *sec* mutants (Plate XLVII, Fig. 3 and 4).

Reversion of the *sec 1* phenotype

After 1.5-h incubation at 37°C *sec 1* cells were transferred to permissive temperature. This postcultivation for 2 h yielded a MT pattern comparable to that at 37°C (Plate XLVIII, Fig. 5). After 4 h of postcultivation all the expected structures were present: cMTs, SPBs, and nMTs in dividing cells. It indicates that the *sec 1* mutation is phenotypically reversible, both in terms of cytokinesis and the microtubular cycle (Plate XLVIII, Fig. 6).

Microtubules in *sec 1* protoplasts

The cells of the *sec 1* mutant were shown to have defect in cMTs when grown at nonpermissive temperature. Accordingly, *sec 1* protoplasts cultured for 1.5 h at nonpermissive temperature contained only single or double SPBs (Plate XLIX, Fig. 7). On the other hand, protoplasts *sec 1* cultivated at permissive temperature contained SPBs, nMTs, and cMTs (Plate XLIX, Fig. 8). Hence it is possible that the *sec 1* gene product interferes with the polymerization of microtubules.

Microtubules in *sec 7* cells and protoplasts

Microtubule patterns similar to wild-type cells were observed in *sec 7* cells growing at 24°C. No alteration in MT arrangement in comparison with 25°C cultivation was found at 37°C. In some mutant cells cMTs were strikingly localized at the cytoplasm periphery, perhaps due to accumulation of GC (Plate L, Fig. 9 and 10). The same experiment was set up with protoplasts of *sec 7* mutant. No substantial difference in MT deployment was observed in protoplasts grown at 24° and 37°C. Both SPBs and cMTs as well as nMTs were observed (not shown).

DISCUSSION

There exists the evidence nowadays that F actin is involved in the directed movement of secretory vesicles in yeasts (Novick and Botstein, 1985). We demonstrated impaired cMTs in the *sec 1* mutant of *S. cerevisiae* under a total blockage of the secretory pathway at its last stage: when the secretory vesicles fuse with the cytoplasmic membrane. Thus cMTs could be involved in secretion at just this stage in concert with the actin filaments by sending some sort of signal to the plasma membrane. However, we cannot exclude the effect of the *sec 1* product might be pleiotropic. On the other hand, having found for *sec 7* that in nonpermissive environment cMTs are not impaired, we are not yet entitled to conclude that they are not important for this phase at all (i.e., transport of secretory material from GC to SV), because a number of genes participate in this step and their products have not been analysed from this aspect thus far.

Using the protoplast system we tried to detect the MT organization step at which the *sec 1* product is switched on. If prior to exposure to nonpermissive conditions cytoplasmic microtubules are distorted and / or depolymerized in fresh protoplasts (Hašek et al., 1986, Jochová-Svobodová et al., 1989), their polymerization would no longer take place at 37°C. Hence we infer that the protein, whose gene has been mutated, could participate in, and is relevant to, MT polymerization. It remains to be explained why cMTs become impaired in *sec 1* cells possessing organized microtubules at the

start of cultivation at 37°C if we have already inferred that the *sec 1* product is of importance for polymerization. Recently a concept of dynamic instability of MTs has been advanced; according to it MTs are not a stationary but a dynamic system in which there is continual polymerization and depolymerization of MTs (Kirschner and Mitchison, 1986; Sammak et al., 1987). Provided this turnover also exists in *sec 1* mutants, polymerization proceeds even in intact cells and in them its mutation-caused alteration might be manifested. In a very few cases cMTs were present in *sec 1* cells at 37°C, however. Should our hypothesis of the involvement of the *sec 1* product in polymerization be valid, then cells with the slow MT turnover (for whatever reason) would have low demands for polymerization and hence microtubular alteration would be manifested only later.

There is another possibility how to explain the reduction of the MT pattern in *sec 1* mutant in nonpermissive conditions. Maybe *sec 1* product (like microtubule-associated protein) is important for stabilization of MTs and microtubules of these mutants are more sensitive for fixation and preparation for indirect immunofluorescence antibody detection in comparison with wild-type cells.

The function of cMTs in intracellular transport of secretory vesicles is a point under discussion. Regarding other models than yeasts (Ericson, 1980; Busson-Mabillot et al., 1982; Bennet et al., 1984a, b) some cell systems need cMTs for transport of SV and organelles, while in others this function has not been proved (Rogalski et al., 1984; Virtanen and Vartio, 1986). By using nocodazole (Jacobs et al., 1988) or cold-sensitive β -tubulin mutants (Huffaker et al., 1988) in *S. cerevisiae*, it was suggested that cMTs are involved neither in polarization nor in intracellular transport of secretory vesicles in spite of the fact that in cold-sensitive β -tubulin mutants in nonpermissive conditions Huffaker et al. (1988) found about 50% less amount of invertase in the external pool than in wild-type cells.

Our results suggest that some *sec* products might assert themselves in the process of secretion through cytoskeletal components — either directly or indirectly in a pleiotropic effect context.

Aknowledgements

I wish to thank Doc. A. Svoboda, Dr. E. Streiblová and Prof. O. Nečas for useful discussion. My thanks are also due to M. Holubář and J. Drápalová for technical assistance.

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Микротубулярная динамика температурночувствительных секреторных мутантов *Saccharomyces cerevisiae*: sec 1 продукт и полимеризация микротубулов

Ляна Брештянска

Резюме. Анализовали отношение цитоплазматических микротубулов к внутриклеточному транспорту у секреторных мутантов *S. cerevisiae* при перmissive и неперmissive температуре непрямой иммуофлуоресценцией с применением моноклонального антитела TU-01 против α -тубулина. Установлено, что у мутанта sec 1, который при 37°C аккумулирует секреторные везикулы, так что угнетается транспорт секреторного материала из секреторных везикул к цитоплазматической мембране, бывают нарушены цитоплазматические, а в некоторых случаях и ядерные микротубулы. После 4-часовой посткультиваии при 24°C измененный фенотип возвращается в первоначальное состояние, клетки делятся и становятся сравнимыми с контрольными. Применяя протопластовую систему, мы установили далее, что продукт, ген которого подвергся мутации, по-видимому, принимает участие в полимеризации микротубулов. У мутанта sec 7, который в неперmissive условиях аккумулирует комплекс Гольджи, так что этим угнетается транспорт секретируемого материала из комплекса Гольджи в выделяющие везикулы, не обнаруживает существенных изменений, которые касались бы длины или количества цитоплазматических микротубулов. В связи с накоплением секретируемого продукта микротубулы в некоторых клетках вытеснены к периферии.

The Modulating Influence of Enkephalins on the Bone Marrow Haemopoiesis in Stress

(stress / haemopoiesis / opioid peptides)

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Received November 27, 1989

Abstract. The influence of enkephalins on haemopoiesis was studied under conditions of stress. Administration of leu-enkephalin and dalargin to mice subjected to immobilization led to inhibition of proliferation and differentiation of haemopoietic precursor cells and to a reduction in the content of cellular elements of erythroid and granulocyte lineages of haemopoiesis. On the contrary, met-enkephalin produced a stimulating influence on the bone marrow haemopoiesis in stress. The regulatory influence of enkephalins is connected with reduction of glucocorticoid production and inhibition of migration to bone marrow of T-lymphocyte regulators of haemopoiesis of the phenotype Lyt-1^+ , Lyt-2^+ and with the direct action on the bone marrow cells. Participation of endogenous leu-enkephalin in the haemopoiesis regulation in stress takes place at an early stage (the first 24 h) of the general adaptation syndrome formation.

Adaptation of the organism to extreme stress occurs with the participation of the haemopoietic system. The functional activity of the latter is under the controlling influence of local regulatory factors (haemopoiesis-inducing microenvironment) whose mobilization under stress occurs with the participation of the central neuro-humoral mechanisms (Goldberg et al., 1987; 1988a, b). In turn, universal stress-realizing systems function in close interaction with the system of endogenous opioid peptides possessing the stress-limiting action on various manifestations of the general adaptation syndrome (Lishmanov, 1986; Meerson, 1987). In particular, opioids can significantly change production of the hypophysis-adrenal system hormones (Gibson et al., 1980; Ixart et al., 1980; Meites, 1984) as well as the state of immunocompetent cells (Foris, 1986) taking part in regulation of haemopoiesis in stress.

Investigations undertaken by us were aimed at studying the influence of enkephalins on the bone marrow haemopoiesis during stress in interaction with other regulatory systems.

MATERIALS AND METHODS

Experiments were carried out on noninbred male mice and (CBA $\times$ C57BL/6) F_1 male mice from the "Central" animal breeding farm (Moscow), weighing 18–20 g. Noninbred mice were subjected to 3–6-h immobilization on the back, and the hybrid mice were subjected to 10-h immobilization. A natural opioid peptide leu-enkephalin, its synthetic analogue dalargin — Tyr-D-Ala-Gly-Phe-Leu-Arg (these preparations were obtained from the Laboratory

Abbreviations: BMCC — bone marrow cell count, CFU — colony forming unit, CIFU — cluster forming unit.

of Peptide Synthesis of the All-Union Cardiological Scientific Centre of the USSR Academy of Medical Sciences) and met-enkephalin (Fluka) were dissolved in distilled water and administered to experimental animals intraperitoneally in a dose of 100 $\mu\text{g/kg}$ once, 6 h, or twice, 3 and 6 h, after the commencement of immobilization. An antagonist of opiate receptors, naloxon (NARCAN), was dissolved in distilled water and administered once intraperitoneally 6 h after the commencement of stress in a dose of 0.2 mg/kg, with subsequent (in 15 min) administration of dalargin, or without it. The influence of endogenous enkephalins on haemopoiesis in stress was studied using an enkephalinase inhibitor, D-phenylalanine (Bodnar et al., 1980), which was dissolved in isotonic saline and injected intraperitoneally once a day in a dose of 300 mg/kg for 7 days before the commencement of immobilization, or after the end of the stress action for 5 days in the same dose. Peripheral blood indices were studied at various times after the start of immobilization. The animals were sacrificed by cervical dislocation. Bone marrow cell count (BMCC) per femur was determined. The myelogram was made on bone marrow smears. The functional state of haemopoietic precursor cells (CFU_{dc}) was assessed upon their cultivation in the plasma clot in diffusion chambers by the method described earlier (Dygai et al., 1985). Besides, the colony-forming activity of the haemopoietic cells was examined *in vitro*. For this purpose, bone marrow cells from the femurs of mice were diluted to a concentration of $10^3/\text{l}$ using semisolid nutrient medium containing 10% foetal calf serum, 5% serum from anaemic mice, 2% conditioned medium of spleen cells, incubated with the mitogen laconos, and 73–83% DI-MEM synthetic medium. Dalargin and naloxon were added to the medium (1/10 of total volume); final concentration of the preparations was 10^{-7} and 10^{-8} M/l, respectively. The cell suspension was incubated in a thermostat at 37°C for one hour. Upon combined addition of naloxon and dalargin, the latter was added to the medium after a 30-min preincubation with naloxon. The cell suspension was placed into each well of a 24-well microtitre plate (Costar, USA) in the amount of 0.5 ml, and incubated for 7 days in a GPI-I incubator (37°C, 100% humidity, 7% CO₂ in air). The colonies (CFUc) in the wells were counted and calculated per 10^6 bone marrow cells after the cultivation.

T-lymphocyte migration to bone marrow was studied using monoclonal antibodies specific for the mouse T-lymphocyte markers — Lyt-1.1 (mouse, μ , k), Lyt-2 (rat, γ 2b, k). Hybridoma supernatants were added to the cell suspension containing 10^9 myelokaryocytes per one l of RPMI-1640 medium. Nontoxic guinea pig serum served as the source of complement. The cytotoxic test was carried out using Trypan blue. The 11-OCS concentration in the animal plasma was determined fluorimetrically in a Hitachi spectrofluorimeter (the width of the outlet 5 nm, excitation wavelength set at 475 nm, and the fluorescence peak at 525 nm).

RESULTS

Stimulation of bone marrow haemopoiesis was observed on the 6th–8th day of investigation in control animals subjected to immobilization and given isotonic saline. The BMCC rose to $19.4\text{--}24.8 \times 10^6/\text{femur}$ (141–156% of the initial level) (Fig. 1).

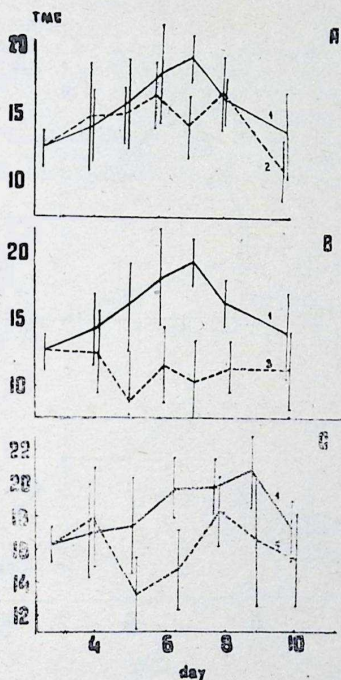


Fig. 1. Changes in bone marrow cell count in noninbred mice subjected to 6-h immobilization against the background of administration of isotonic saline (1), leu-enkephalin (2), dalgargin (3) and D-phenylalanine (4) before (C) and after (A, B) the stress action; abscissa — time after immobilization; ordinate — number of cells ($\times 10^6/\text{femur}$). Confidence intervals at $P = 0.05$.

An increase in total bone marrow cellularity was preceded by an increase in its colony- and cluster-forming activity from the 5th day of investigation up to 408% (CFU_{dc}) and 226% ($ClFU_{dc}$) of the initial level (Fig. 2). Analysis of the quantitative composition of colonies identified in total preparations demonstrated that immobilization stimulated formation of not only granulocytic, macrophagic and granulocytic-macrophagic, but also of erythroid

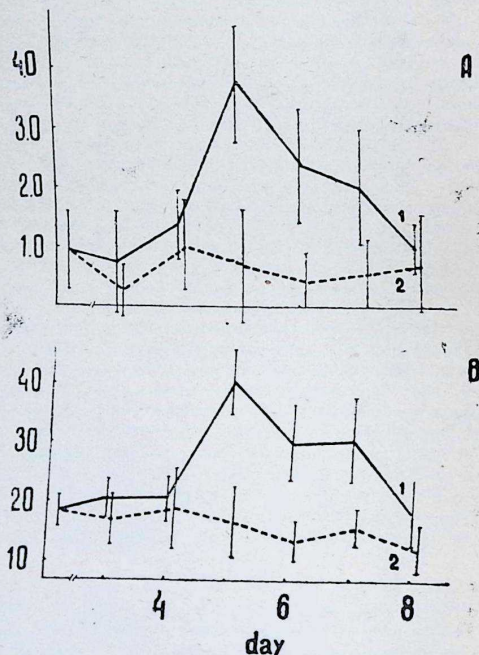


Fig. 2. Cloning efficiency of CFU_d (A) and $ClFU_{dc}$ (B) from the bone marrow of (CBA $\times$ C57BL/6) F_1 mice subjected to 10-h immobilization and given isotonic saline (1) and dalargin (2) 6 h after the commencement of stress action. Abscissa — time after the commencement of immobilization; ordinate — CFU_d and $ClFU_{dc}$ content per 10^5 myelokaryocytes. Confidence intervals at $P = 0.05$.

colonies not observed in cultivation in the plasma clot of bone marrow from nonimmobilized mice. An increase of haemopoietic precursor cells committed in the direction of erythro- and granulomonocytopoiesis in the bone marrow of immobilized animals was accompanied with stimulation of different haemopoietic nodes detected in analysing the myelograms. Thus, the content of immature forms of neutrophilic granulocytes increased on the 5th–7th day of experiment to 137–194% of the initial level in different series of experiments, and constituted 4.6 ± 0.4 to $5.1 \pm 0.4 \times 10^6$ /femur. There was also a rise of bone marrow band forms and polymorphonuclear neutrophilic granulocytes on the 4th–8th day after the start of immobilization up to $11.6 \pm 0.5 \times 10^6$ /femur (175% of the initial level), and of erythrokaryocytes of different degree of maturity up to $4.9 \pm 0.6 \times 10^6$ /femur (245% of the initial level). An increase in the peripheral blood formed elements was a regular consequence of bone marrow hyperplasia in stressed animals. Thus, the content of polymorphonuclear neutrophilic granulocytes rose on the 5th–6th

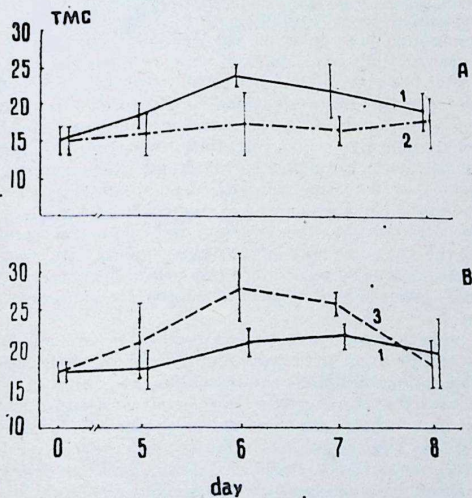


Fig. 3. Changes in bone marrow cell count in mice subjected to 6-h (A) and 3-h immobilization (B) against the background of isotonic saline (1), leu-enkephalin (2) and met-enkephalin (3) administration. Abscissa — time after the start of immobilization; ordinate — cell content ($\times 10^6$ /femur). Confidence intervals at $P = 0.05$.

day of experiment up to $6.08 \pm 0.65 \times 10^6/l$ (147% of the basic figure); on the 6th day after the start of immobilization there was monocytosis up to $2.36 \pm 0.38 \times 10^9/l$ (236% of the initial level). Erythropoiesis stimulation was confirmed by the development of reticulocytosis from the 3rd day of study (up to $84.8 \pm 4.9\%$ with the basic figure of $47.7 \pm 4.1\%$), of erythrocytosis on the 7th day of experiment (up to $8.5 \pm 0.2 \times 10^{12}/l$; 110% of the initial level).

The picture of bone marrow haemopoiesis changed when leu-enkephalin or dalargin was administered to experimental animals 6 h after the beginning of immobilization. In particular, stress-induced bone marrow hyperplasia was less pronounced in animals given leu-enkephalin than in control animals. Dalargin injection led to complete suppression of bone marrow haemopoiesis stimulation in stress, up to development of moderate hypoplasia on the 5th day of investigation (Fig. 1).

A change in the adaptive response of the haemopoietic tissue under the effect of leu-enkephalin and dalargin was connected with their influence on processes of proliferation and differentiation of precursor cells and morphologically differentiated bone marrow cell elements. Thus, no increase in colony- and cluster-forming bone marrow activity occurred in animals given dalargin 6 h after the start of immobilization (Fig. 2). It is mostly colonies of macrophage type that were identified in the total preparations; no erythroid colonies were found. With the mentioned changes the bone marrow of immobilized animals given opioid peptides displayed a fall in the absolute number of cell elements belonging to erythroid and granulocytic haemopoietic lineages. Thus, in comparison with the control group, under the effect of dalargin a reduction in the content of immature and mature neutrophilic granulocytes occurred on the 5th—7th day, and upon administration of leu-enkephalin on the 6th—7th day of the experiment. The total number of erythrokaryocytes proved to be significantly below the corresponding control values in animals given leu-enkephalin and dalargin on the 6th—7th day of the experiment.

Changes in bone marrow haemopoiesis indices in stressed animals given leu-enkephalin and dalargin influenced the peripheral blood indices as well. Thus, both preparations inhibited the development of neutrophilic leukocytosis, monocytosis and erythrocytosis. On the contrary, administration of leu-enkephalin and its synthetic analogue led to moderate erythropenia on the 4th (down to 80.5%) and the 5th—6th day of the experiment (down to 70% of the basic level), respectively. Besides, dalargin prevented development of reticulocytosis throughout the observation period. It should be noted that, in contrast to leu-enkephalin and dalargin, met-enkephalin was capable of enhancing the adaptive response of the haemopoietic tissue. Whereas in control animals subjected to 3-h immobilization, there was only a short-term (on the 7th day) increase in total bone marrow cellularity, up to 127% of

the initial level, met-enkephalin administered twice to the animals after the end of immobilization led to an increase in total myelokaryocyte count on the 5th—7th day of study — up to 148% of the initial level on account of activation of erythro- and granulocytopoiesis (Fig. 3).

Activation of the system of endogenous opioid peptides also produced changes in the indices of bone marrow haemopoiesis in stress. We recorded an increase in the concentration of endogenous leu-enkephalin up to 904.6 pg/ml (338% of the initial level) in the plasma of mice subjected to 6-h immobilization 3 and 6 h after the commencement of stress action. The course of administration of the enkephalinase inhibitor D-phenylalanine before the start of immobilization led to inhibition of enzymatic degradation of endogenous leu-enkephalin and to a marked increase in its content in the plasma of stressed mice during the first 24 h of investigation. The concentration of that enkephalin in the plasma of animals given D-phenylalanine exceeded 6.3 times that observed after the administration of isotonic saline 6 h and 27 times that seen 12 h after the commencement of immobilization. At the same time, no stimulation of haemopoiesis occurred in animals given D-phenylalanine before the immobilization (Fig. 1). It should

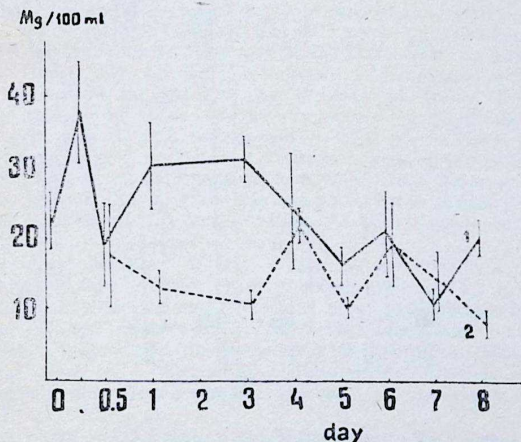


Fig. 4. Changes in the content of 11-OCS in the blood plasma of mice subjected to 6-h immobilization and given isotonic saline (1) and dalargin (2) 6 h after the commencement of stress action. Abscissa — time after the start of immobilization; ordinate — 11-OCS concentration, $\mu\text{g}/100\text{ ml}$. Confidence intervals at $P = 0.05$.

be noted that the course of administration of D-phenylalanine started after the immobilization failed to alter the nature of the haemopoietic tissue adaptive response. The number of nucleated bone marrow cells in mice of this experimental group showed no significant difference from that of the control immobilized animals at all intervals of investigation, with no suppression of granulocytopoiesis stimulation.

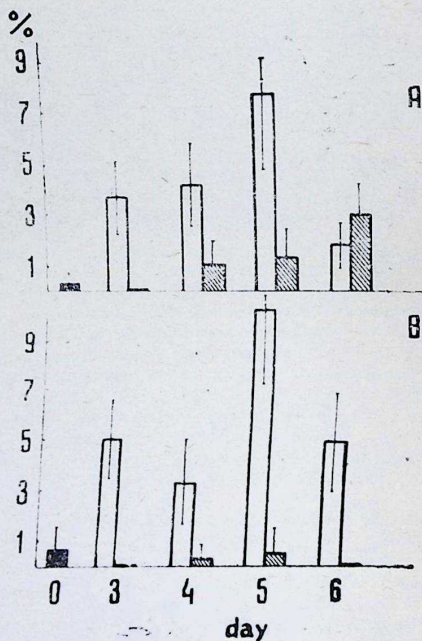


Fig. 5. Dynamics of Lyt-1 (A) and Lyt-2 (B) antigen expression on the bone marrow lymphocytes of (CBA x C57BL/6)F₁ mice subjected to 10-h immobilization and given isotonic saline (□) or dalargin (▨) 6 h after the commencement of stress action. Abscissa — time after the beginning of immobilization; ordinate — index of antibody toxicity. Confidence intervals at $P = 0.33$.

Table 1. Efficiency of CFU_c cloning (per 10⁶ bone marrow cells) of the bone marrow of (CBA × C57BL/6)F₁ mice on the 5th day after a 10-h immobilization in cultures with dalargin, naloxon, or both ligands ($\bar{X} \pm m$; P)

Without addition of opiate receptor ligands	Total number of CFU _{dc} 12.0 ± 2.4
Dalargin 10 ⁻⁷ M/l	1.6 ± 0.8 <0.01
Naloxon 10 ⁻⁸ M/l	5.6 ± 2.4 >0.05
Naloxon + dalargin	6.0 ± 0.8 <0.05

To ascertain the possible mechanisms of the regulatory action of enkephalins on the bone marrow haemopoiesis, we investigated the influence of dalargin on the glucocorticoid content in the plasma and of T-lymphocyte regulators of haemopoiesis in the bone marrow of experimental animals. Immobilization led to a marked increase in the 11-OCS content in the blood plasma of mice 6 h after the start of stress action, the increase being retained up to 3 days of study; this preceded development of bone marrow hyperplasia (Fig. 4). Besides, on the 3rd day after the start of stress action the bone marrow of immobilized animals showed an increase in the number of T-lymphocytes with the Lyt-1⁺, Lyt-2⁺ phenotype (Fig. 5). At the same time, in mice given dalargin 6 h after the commencement of immobilization, no increase in the content of plasma glucocorticoids took place at subsequent observation periods (Fig. 4); there was also a reduced migration of T-lymphocytes expressing Lyt-1 and Lyt-2 antigens to bone marrow in comparison with that observed in the control group (Fig. 5).

The possibility of direct dalargin action on the bone marrow nuclears was investigated upon addition of the preparation to the culture medium for cloning in diffusion microchambers of the bone marrow obtained from animals on the 5th day after the start of the stress action. The efficiency of CFU_{dc} and CUFU_{dc} cloning was reduced (dose-dependent effect) until the clone growth was completely suppressed in the medium containing 10⁻⁹ M/l of the preparation. An analogous effect of dalargin was observed, when added to the culture medium *in vitro* (Table 1).

The specificity of the action of opioid peptides on the haemopoietic tissue was studied using an antagonist of opiate receptors, naloxon, whose administration 15 min before dalargin injection reduced the suppressive action of the latter on haemopoiesis in stress. An analogous effect of naloxon was observed *in vitro* (Tables 1, 2).

Table 2. Dynamics of indices of bone marrow haemopoiesis ($\times 10^6/\text{femur}$) in mice subjected to immobilization and given isotonic saline (I), dalgargin (II), naloxon (III), naloxon + dalgargin (IV) 6 h after the start of immobilization ($X \pm m$; P)

	Periods of investigation, days	Bone marrow cell count	Immature neutrophilic leucocytes	Erythroid cells
	Before immobilization	17.1 ± 0.7	4.1 ± 0.3	1.7 ± 0.3
I	5	18.1 ± 0.9 0.2	5.6 ± 0.4 0.02	1.6 ± 0.2 0.5
	6	24.1 ± 1.5 0.01	5.3 ± 0.4 0.05	3.9 ± 0.5 0.01
	7	19.9 ± 1.2 0.05	5.3 ± 0.6 0.05	1.9 ± 0.5 0.5
	8	17.9 ± 0.8 0.5	4.5 ± 0.4 0.05	1.7 ± 0.3 0.5
II	5	14.3 ± 1.3 0.05	4.2 ± 0.4 0.5	0.8 ± 0.1 0.02
	6	15.5 ± 1.2 0.2	4.1 ± 0.2 0.1	1.1 ± 0.3 0.1
	7	15.5 ± 1.0 0.2	3.4 ± 0.4 0.1	0.9 ± 0.2 0.05
	8	13.5 ± 1.2 0.05	3.0 ± 0.2 0.01	0.7 ± 0.2 0.02
III	5	17.1 ± 1.8 0.5	4.5 ± 0.4 0.2	1.9 ± 0.5 0.5
	6	17.3 ± 0.8 0.5	4.3 ± 0.3 0.5	2.8 ± 0.4 0.05
	7	18.4 ± 1.4 0.2	4.2 ± 0.2 0.5	1.8 ± 0.4 0.5
	8	14.0 ± 1.7 0.1	4.2 ± 0.3 0.5	1.6 ± 0.6 0.5
IV	5	18.4 ± 1.0 0.5	5.4 ± 0.5 0.05	1.8 ± 0.5 0.5
	6	16.9 ± 0.9 0.5	4.7 ± 0.3 0.5	1.4 ± 0.3 0.5
	7	16.1 ± 1.3 0.5	4.3 ± 0.3 0.5	1.3 ± 0.2 0.5
	8	19.3 ± 1.8 0.5	4.6 ± 0.5 0.5	1.7 ± 0.4 0.5

DISCUSSION

Thus, an increase in the concentration of exogenous or endogenous enkephalins in the blood plasma of experimental animals led to changes in the nature of the adaptive response of haemopoietic tissue. Leu-enkephalin and its synthetic analogue dalgargin possessed a suppressive, and met-enkephalin a

stimulating influence on the bone marrow haemopoiesis during immobilization stress. The regulatory influence is produced by enkephalins through the glucocorticoid and T-lymphocyte control mechanisms of the proliferation and differentiation of haemopoietic cells. Homing into bone marrow of T-lymphocyte regulators is known to occur in stress under the effect of glucocorticoids (Dygai et al., 1985, 1986); T-lymphocyte subpopulations stimulating and normalizing proliferation and differentiation of the haemopoietic precursors consecutively migrate to the haemopoietic tissue (Goldberg et al., 1988c). Diminished migration of T-lymphocyte regulators under the effect of dalargin, initiating the haemopoietic processes, can lead to suppression of the erythro- and granulomonocytopoiesis in immobilized mice which received the opioid peptide. Apart from the mediated influence, a direct effect of enkephalins on the bone marrow cells, leading to changes in the functional activity of the haemopoietic precursors, cannot be excluded. This action may be realized through the opiate receptors on haemopoietic precursor cells. The action of opioids at the cellular level is connected with reduction of the intracellular cAMP content (Blume and Boone, 1979; Schoffemeer et al., 1987) which can determine the intensity of proliferation and differentiation of blood cells (Hadden et al., 1979). The possibility cannot be excluded that the influence of opioid peptides is realized through the receptors on the cellular elements forming haemopoiesis-inducing micro-environment (T-lymphocytes, macrophages, stromal mechanocytes) on account of changes in the production of short-distance humoral factors regulating proliferation and differentiation of haemopoietic precursor cells.

Involvement of opiate receptors in the manifestation of the enkephalin effect is confirmed by the reduction of its expression under the influence of naloxon, an opiate receptor antagonist *in vivo* and *in vitro* (Tables 1, 2). At the same time naloxon itself inhibited the development of bone marrow hyperplasia under conditions of immobilization stress (Table 2). An explanation of this effect can be sought in the differently directed influence of different enkephalins on haemopoiesis in stress. Naloxon, serving primarily as an antagonist of μ -opiate receptors, with which met-enkephalin interacts specifically, can inhibit the stimulating influence of the latter on bone marrow haemopoiesis. At the same time, blocking the action of leu-enkephalin, expressed through the opiate receptors δ -, naloxon diminishes the suppressive effect of opioid peptides on haemopoiesis in stress.

Participation of endogenous enkephalins in the formation of the haemopoietic tissue adaptive response is expressed at an early stage of the general adaptation syndrome. An accumulation of an endogenous leu-enkephalin during the first 24 h of stress under the influence of D-phenylalanine, an enkephalinase inhibitor, serves in favour of this assumption. When the preparation is administered after the end of the stress action, it fails to alter the haemopoietic tissue reactivity.

Final solution of the problem concerning the physiological role played by endogenous opioid peptides in the regulation of bone marrow haemopoiesis under normal conditions and under extreme stress actions will be the subject of further investigations.

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Модулирующее влияние энкефалинов на кроветворение в костном мозге при нагрузке

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Резюме. Изучали влияние энкефалинов на кроветворение в условиях нагрузки. Введение лей-энкефалина и даларгина иммобилизованным (обездвиженным) мышам угнетало пролиферацию и дифференциацию гемопоэтических клеток-предшественников и понижало содержание клеточных элементов эритроидной и гранулоцитарной линий кроветворения. Наоборот, мет-энкефалин оказывал стимулирующее действие на кроветворение в костном мозге при нагрузке. Регулирующее влияние энкефалинов связано с понижением продукции глюкокортикоидов и с угнетением миграции Т-лимфоцитов-регуляторов кроветворения с фенотипами Lyt-1^+ , Lyt-2^+ и с прямым действием на клетки костного мозга. Участие эндогенного лей-энкефалина в регуляции кроветворения при нагрузке наступает в ранней стадии (в течение первых 24 часов) образования общего адаптационного синдрома.

Cataract-related Changes in Protein Aggregates of Human Lens Studied by Ultracentrifugation*

(human cataract / infrared spectra)

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Received November 6, 1989

Abstract. In human lens, cataract development causes an increase in the amount of protein aggregates. Their specific density of 1.25 g/ml is much higher than that of 1.14 g/ml and 1.06 g/ml obtained for water-soluble proteins. During the formation of water-insoluble aggregates their size varies up to some micrometers. Infrared spectra of water-soluble protein and in the aggregates confirm that the content of bound water in aggregates decreases.

In the lens, there are different soluble proteins, designated as different types of crystallins. The α -crystallins have the highest molecular weight (400—1200 kDa) (de Jong, 1981; Bloemendal, 1982), the β -crystallin proteins can associate to form diverse mixtures of homo- and hetero-polymers of various size (Barbers et al., 1984; Hogg et al., 1986; Slingsby et al., 1988) and have molecular weights 40—200 kDa, and γ -crystallin proteins are characterized by monomeric molecular weights of about 20 kDa (Meakin et al., 1985; Moormann et al., 1985; Summers et al., 1986). The insoluble protein fraction has not been studied frequently. An increase in the amount of insoluble protein reflects the decrease in soluble protein with the aging of the lens (Spector et al., 1975). In 1973, Spector and Rothschild found the presence of aggregates with molecular weights greater than 50×1000 kDa using an agarose of large pore size (Bio-Gel A-50 m). Relative amounts of these fractions as percentage of total α -crystallin increased with age both in the cortex and the nucleus (Harding and Dilley, 1976). High-molecular-weight protein aggregates of α -crystallin have been isolated from cataractous human lens (Harding and Dilley, 1976; Roy and Spector, 1978a). Moreover, aggregates up to 500 nm across in human lens have been observed by electron microscopy (Liem-The et al., 1975). Philipson (1973) has identified dense clusters of 50—100 nm in the lens nucleus of human cataracts.

The water-insoluble proteins of the human ocular lens are composed of both low- and high-molecular-weight components (Truscott and Augusteyn, 1977; Roy and Spector, 1978a). Roy and Spector (1978a) have shown that the 20—27-kDa fraction is the component containing 30% of water-soluble protein with 20% represented by the 11-kDa component. The absence of any free NH_2 -terminal residues in the water-insoluble fraction has been interpreted as absence of unmodified γ -crystallin. The relative concentration of total water-insoluble protein generally increases in human and exper-

Abbreviations: SDS — sodium dodecyl sulphate.

* This work was supported by Polish Grant RPB — RR. I. 10/3.

imental cataracts and could contribute to development of these cataracts (Giblin et al., 1978; Cenedella and Bierkamper, 1979).

In fact, little is known as yet concerning the composition of these protein aggregates. For the first time we have obtained very large protein aggregates $>6 \mu\text{m}$, without any other cell components, by ultracentrifugation in sucrose density gradient. These aggregates are many times greater than any aggregates previously observed. The sucrose gradient centrifugation has been done for human lens proteins after dissolving in SDS solution (Takemoto and Azari, 1977). These aggregates have been additionally isolated from totally guanidine-solubilized lens proteins and not just from the whole lens homogenate.

MATERIALS AND METHODS

Human lenses cryoextracted during the surgical procedure were used as the material. The operation for the cataract consisted of opening of an eyeball followed by cryoextraction of the lens. After macroscopic examination, the lens was placed in a polyethylene tube and frozen at -20°C . To determine the severity of human lens cataract, a special factor (so-called Medical factor M) was introduced by Prof. H. Żygulska-Mach (Twardowski et al., 1988). Senile cataracts subjected to examination were divided into 9 groups according to the degree of opacity which was regarded as an indicator of cataract development. This division is more precise than that usually used in clinical practice. The estimation of the degree of opacification was based on an examination of the eye in the beam of a slit-lamp under mydriasis, as well as on the microscopic observation of the lens during the operation.

For further studies, the whole lens was placed in 0.1 M Tris-HCl buffer, pH 7.2, homogenized with a knife homogenizer rotating at 60 rev/min for 60 sec at 20°C . The homogenate was placed on the top of a $8.5 \text{ cm} \times 2.5 \text{ cm}$ tube with 0–70% sucrose density gradient and then it was centrifuged at 35,000 rev/min for 16 h at 20°C on the angle-head rotor of a UP-65 ultracentrifuge.

After centrifugation the tube was taken out very carefully and the sample was fractionated from the top of the tube by displacement with a dense medium iodoethane which was pumped in at the bottom. Then the fractions were collected with a fraction collector. For each fraction the protein concentration was determined using the Bradford method (Bradford, 1975) and the sucrose concentration was determined with a sugar refractometer using α -bromonaphthalene as a standard.

Slab gel electrophoresis ($12 \text{ cm} \times 13 \text{ cm} \times 0.15 \text{ cm}$) was performed on 5% stacking gel and 12% separating gel according to Weber and Osborne (1969). Reduced samples of protein ($15 \mu\text{g}$) in 1% sodium dodecyl sulphate and 5% mercaptoethanol for 40 min at 100°C were used. The protein bands were detected by staining with Coomassie blue.

For the infrared measurements, the water was evaporated by the air flow at room temperature and solid-state thin films were measured on Irtran 2 plates. Infrared spectra were measured in the region at $3700-1205\text{ cm}^{-1}$ with a Specord IR 75 spectrophotometer with a Commodore C-64 computer off line, and analysed using an IBM XT computer.

RESULTS

Some examples of homogenate separations of human opaque lenses by centrifugation are presented in Fig. 1. The densities of the banded samples are the same for lenses in different stages of cataract development and they are: 1.06 g/ml for band I, 1.14 g/ml for band II and 1.25 g/ml (maximal value 1.28 g/ml) for band III. The band III fraction is connected to large-size protein aggregates. For higher values of medical factor it is quite distinct and it has a brown colour and in each case is opalescent. The fractions I and II are completely transparent and colourless.

The increase in the medical factor with cataract development is proportional to the increase in the protein aggregates in percent of total protein in

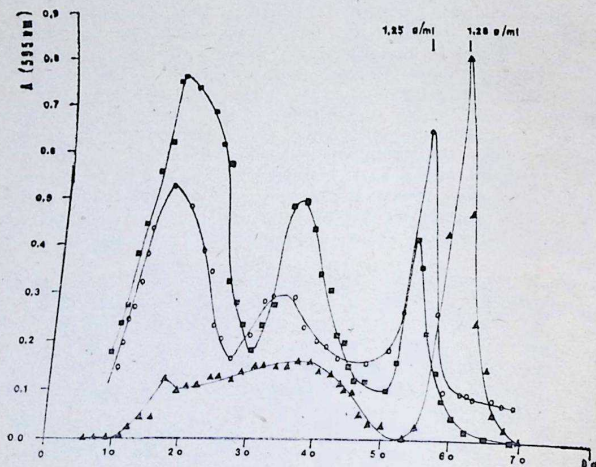


Fig. 1. Separation of homogenate of human lens in a sucrose density gradient for different medical factors: $\blacksquare$ - $M = 1$, $\circ$ - $M = 4$, $\blacktriangle$ - $M = 6$.

the lens (Fig. 2). The total protein, as well as the protein aggregates in percent of total protein, were found by measuring respective surfaces under line (Fig. 1) using a planimetry method. An examination of the effect of aging upon the distribution of the insoluble proteins has been reported many times. However, in this experiment, the medical factor determined much better the senile cataract development than the age of individual lens.

Protein aggregates from different kinds of human lenses are presented in Plates LI, LII, LIII, Fig. 3. Among many small aggregates exist the big ones with a size of about a few micrometers. Some of them are completely black, but usually they are yellow or green.

Figures 4—6 present the infrared spectra of thin protein films from human lens. Fig. 4 shows the infrared spectra of protein fractions I and II obtained after centrifugation. Both spectra are very similar and the differential infrared spectrum between them has no distinct bands, showing a small decrease in water content as also observed in the $1700-1205\text{ cm}^{-1}$ region in the protein

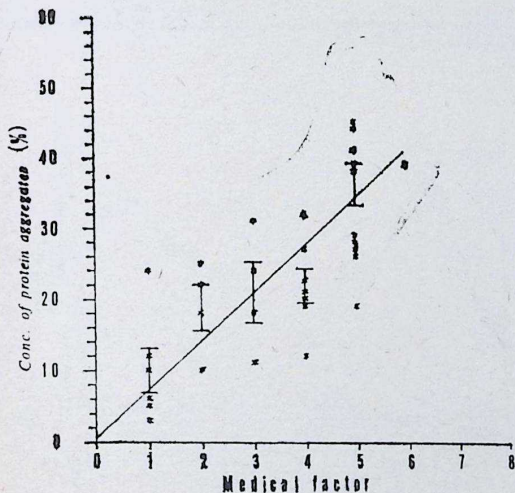


Fig. 2. Relationship between the amount of protein aggregates in percent of total protein in the lens and medical factor.

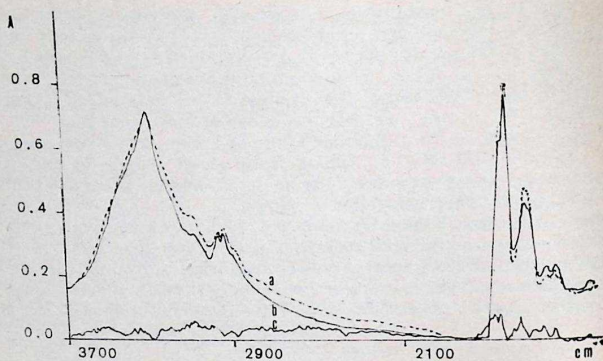


Fig. 4. Infrared spectra of protein fraction: a — I and b — II, obtained from centrifugation and the infrared difference spectrum $c = a - b$.

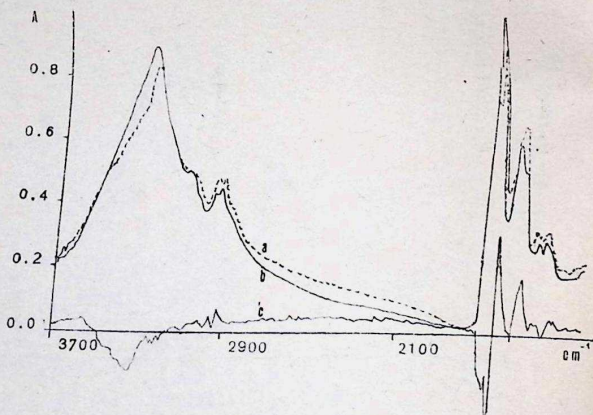


Fig. 5. Infrared spectra of protein fraction: a — I and b — III, obtained from ultracentrifugation of human lens homogenate ($M = 5$) and the infrared difference spectrum $c = a - b$.

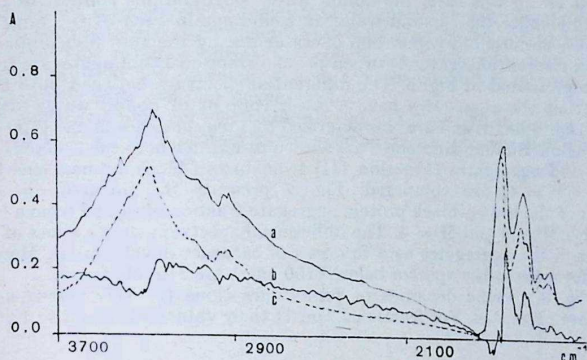


Fig. 6. Infrared spectra of protein fraction: a — I and b — III, obtained from ultracentrifugation of brown-black human lens homogenate ($M = 4$) and the infrared difference spectrum $c = a - b$.

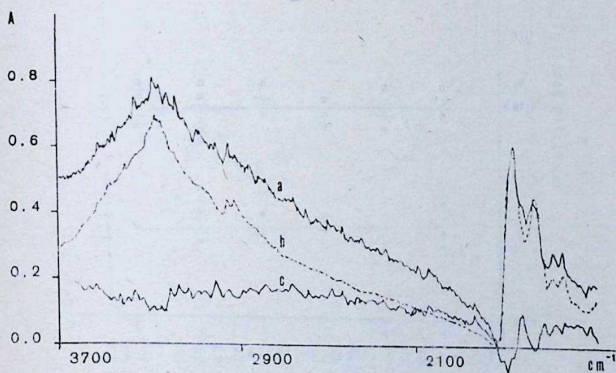


Fig. 7. Infrared spectra of protein fraction III (aggregates) for brown-black human lenses for medical factors: a — $M = 6$, b — $M = 4$, and c — their differences spectrum $a - b$.

fraction II. In this case, the bound water surroundings content of protein is much smaller than bound water surroundings in each of the centrifuged fractions, because of evaporation of water during the thin film preparation.

Much clearer differences between protein fraction I and aggregate fraction III are presented in Fig. 5. The differential spectrum between both spectra shows that the aggregates have a lower content of bound water (decrease of 3322 cm^{-1} band) and are characterized by great changes in the $1750\text{--}1205\text{ cm}^{-1}$ region. Similar differences are shown in Fig. 6 where the spectra of fraction I and aggregates (Fraction III) from brown-black human lens homogenate ($M = 4$) are compared. Fig. 7 presents the infrared absorption spectra of the brown-black protein aggregate fraction obtained from a human lens with $M = 6$ and $M = 4$. The differential spectrum shows a loss of water content in the aggregates as a function of cataract development. Moreover, the differences in the spectra below 1700 cm^{-1} are also observed.

In Fig. 8, specific densities of protein fractions (I—III) versus medical factor are presented. For fraction I and II their values are equal to 1.06 g/ml

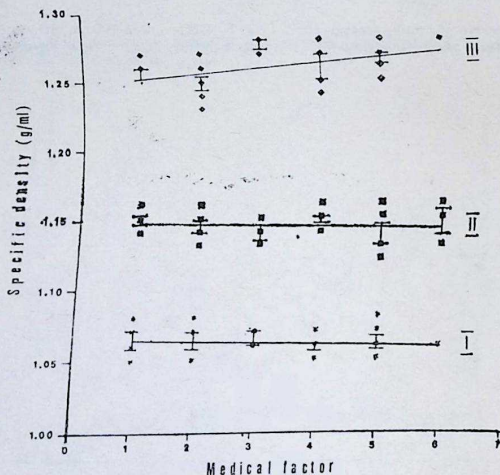


Fig. 8. Specific density of protein fractions I, II and III after ultracentrifugation of homogenate of human lens in a sucrose density gradient for different medical factors.

and 1.145 g/ml, respectively. It seems that the specific density for fraction III increases with cataract development from 1.25 g/ml for $M = 1$ to 1.27 g/ml for $M = 6$. This result confirms the conclusion made from the infrared measurements that the decrease in bound water content in protein aggregates proceeds with cataract development.

SDS-polyacrylamide gel electrophoresis at pH 7 of lens insoluble (aggregates) fractions shows 7 protein bands. The aggregate protein distribution is

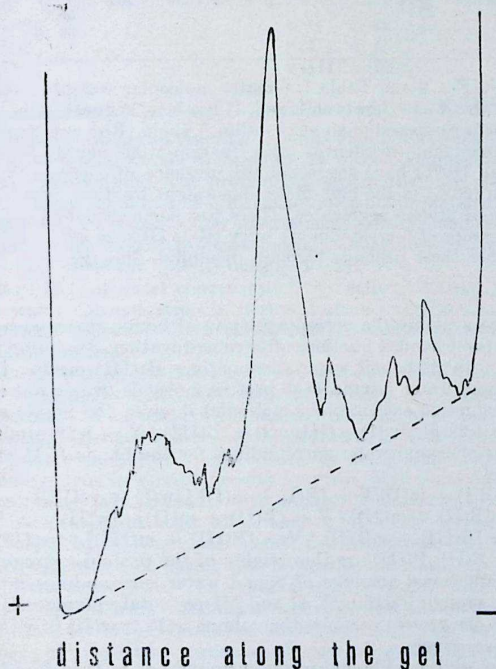


Fig. 9. SDS-polyacrylamide gel electrophoresis of fraction III (aggregates) of human lens ($M = 5$) after ultracentrifugation in a sucrose density gradient.

Table 1. Distribution of protein in human lens aggregates

Apparent molecular weight (kDa)	Percentage (%)
62.5	6
53.5	8
48.5	
34	6
28	55
25.5	
12.5	25

presented in Fig. 9 and Table 1. Greater molecular weights than those presented in Table 1 have not been found. It has been suggested that the 11-kDa polypeptide is composed of an α -crystallin A chain (Roy and Spector, 1978b). In this experiment we identify this fraction with our 12.5-kDa fraction. Giblin et al. (1978) have suggested the presence of α -crystallin (40%) and β -crystallin (60%) in the high-molecular-weight fraction, which is probably a precursor of protein aggregates. There was some evidence suggesting that the $-SH$ groups of β -crystallin are very labile (Dische et al., 1956). It would bind together these proteins through disulphide linkage.

DISCUSSION

It is easy to evaluate the percentage (v/v) of bound water surroundings in each of the three banded fractions after centrifugation. Assuming that protein aggregates with its bound water surroundings B (III) occupy the whole V without bound water around each protein molecule (we do not evaluate the bound water inside each protein molecule) it gives the maximal density ρ (III_{max}) = 1.28 g/ml: (1) ρ (III_{max}) = B(III) / V = 1.28 g/ml.

The amount of bound water surroundings for the bands I, II and III may be evaluated:

$$(2) \rho(I) = [B(I) + m(I)] / V = [B(I) + m(I)] / [w(I) + v(I)]$$

$$(3) \rho(II) = [B(II) + m(II)] / V = [B(II) + m(II)] / [w(II) + v(II)]$$

$$(4) \rho(III) = [B(III) + m(III)] / V = [B(III) + m(III)] / [w(III) + v(III)],$$

where B(I), B(II), B(III) are the weights of the protein aggregates reduced because of additional amounts of bound water surroundings m(I) or m(II) or m(III) in protein fractions I, II and III per total volume v(I) or v(II) or v(III), while the protein occupies the volume w(I) or w(II) or w(III) in these fractions, respectively.

Weights of additional bound water surroundings m(I) in fraction I, m(II) in fraction II and m(III) in fraction III per total volume increase, while weights of protein decrease in relation to the aggregate fraction. In each case total volume V is equal to the sum of protein volume w(I) or w(II) or

$w(\text{III})$ and bound water surroundings volume $v(\text{I})$ or $v(\text{II})$ or $v(\text{III})$, respectively.

$$(5) \varrho(i) = B(i) / w(i),$$

where in this case $w(i)$ for $i = 1, 2$ and 3 is the volume occupied by protein aggregates without its bound water. The bound water inside the protein molecule has not been determined by us.

The solution of the above equations gives the following results:

$$v(\text{I}) = 78\%$$

$$w(\text{I}) = 22\%$$

$$v(\text{II}) = 50\%$$

$$w(\text{II}) = 50\%$$

$$v(\text{III}) = 7\%$$

$$w(\text{III}) = 93\%$$

and

$$V = w(\text{I}) + v(\text{I}) = w(\text{II}) + v(\text{II}) = w(\text{III}) + v(\text{III}) = W(\text{III}) = 100\%.$$

It means that proteins for fraction I occupy 22% of total solution volume, for fraction II 50% and for the fraction of the protein aggregates (III) they occupy 93% of the volume. The protein aggregates that are characterized by maximal density equal to 1.28 g/ml do not have bound water surroundings inside the complex. Of course, water environment exists around the aggregate but not around each of the proteins forming an aggregate. In the whole volume, V water gives the specific density 1 g/ml.

The result of 78% of water concentration in fraction I corresponds well to about 70% water concentration in normal human lens (Racz et al., 1979). A small difference between these values may be connected with the other types of proteins in lens.

On the basis of our results it is possible to propose a mechanism of changes in lens protein aggregates in which cataract develops. We suggest two independent processes: (1) increase in the number and size of protein aggregates, (2) nonenzymatic changes inside each aggregate as a function of time.

The first process is shown in this work by using ultracentrifugation of homogenized eye lens in sucrose density gradient. Moreover, specific density of the aggregates slowly increases with cataract development.

Nonenzymatic changes start after binding of single crystallins in proportions revealed by SDS-polyacrylamide electrophoresis. The high density of aggregates suggests that during this process bound water is removed from the inside of the aggregates. The infrared spectra confirm this observation. Moreover, aggregates from the human lens with the highest medical factor are characterized by lower content of bound water. It seems that conformational changes and oxidation processes on the protein surface in aggregates follow step by step. Aggregates, which were observed under a light microscope (magnification $175\times$), changed their colour. In a carboxygen atmosphere (95% O_2 , 5% CO_2), changes of colourless shapes through green to yellow may be observed. Reduction of the aggregates by strong acid solution created colourless aggregates. Addition of a drop of 1 M NaOH to

the aggregates makes them yellow. That process is connected with the redox reaction of quinones (Zigman, 1971), probably universal for other proteins which have aromatic amino acid residues. We believe that these changes lead to the formation of melanin and the black aggregates contain some amount of it. Colour aggregates may represent intermediate protein complexes in the biochemical cycle of melanin formation. The increase in infrared background and changes in the $1700-1200\text{ cm}^{-1}$ region are characteristic of the non-specific spectra of different melanin types (Filip et al., 1974). The chemical procedure for preparing melanin from biological material (Korytowski, 1982) yields some micrograms of a black powder from cataractous human lenses. It is extensively studied in our laboratory. Such complex is very difficult to dissolve without using an extremely aggressive chemical method. This is also characteristic of melanin complexes.

Nonenzymatic browning of lens proteins *in vivo* during incubation with reducing sugars leads to the formation of fluorescent yellow pigments (Monnier and Cerami, 1981). The phenomenon called nonenzymatic browning or Maillard reaction may be an alternative explanation for nonenzymatic changes inside each aggregate as a function of time.

Acknowledgement

The authors thank Prof. H. Żygulska-Mach (Clinic of Ophthalmology, Medical Academy, Cracow) for kindly supplying human lenses.

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Исследования путем центрифугирования изменений, родственных катакте, в протениновых агрегатах в хрусталиках глаз у людей

Я. Твардовски, Дорота Гойа

Резюме. В хрусталиках человека развитие серого бельма вызывается повышением количества протениновых агрегатов. Их специфическая густота 1,25 г/мл гораздо выше, чем специфическая густота 1,14 г/мл и 1,06 г/мл, наблюдаемая в случае водорастворимых протенинов. В течение образования агрегатов, не растворимых в воде, их размеры разнятся и на несколько микрометров. Инфракрасные спектры водорастворимых протенинов и протенинов в агрегатах подтверждают тот факт, что содержание связанной воды в агрегатах понижается.

Pretreatment with Thymus Cell-conditioned Medium Enhances the Whole-animal Survival of Mice Exposed to Ionizing Radiation

(conditioned medium / radioprotection / gamma radiation / survival)

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Received November 27, 1989

Abstract. Pretreatment with cell-free supernatant of thymus cell-conditioned medium (TCCM) protects mice in a dose-dependent manner from the lethal effect of ionizing radiation. A single dose of TCCM injected i.p. in a volume of 1.2 ml 8 h before lethal gamma irradiation with a dose of 9 Gy ($LD_{95/30}$) protected 36% of C57BL/6 mice. Four doses of TCCM injected i.p. in a volume of 0.8 ml once a day (i.e., during four days), protected 50% of C57BL/6 mice. Application of five-fold concentrated supernatant of conditioned medium (cTCCM) in a volume of 1 ml i.p. 8 h before irradiation with a dose of 9 Gy protected 75% of mice. On the other hand, no therapeutic effect of different doses of the supernatant fluids from thymus cell cultures was found. Our observations suggest that media conditioned by thymus cells can mediate a radioprotective effect.

In the last years within the search for therapeutic and prophylactic radioprotective substances, attention has been paid to nonsulphydryl naturally occurring compounds including vitamins, selenium, etc., or immunomodulators and cell-growth factors. Among the studies those concerning endogenous protective systems capable of protecting against radiation and chemical damage (Weiss and Simic, 1988) seem to be of great importance. The above mentioned compounds attract attention for two main reasons: first, since proliferation and differentiation of haemopoietic and lymphoid cells are mediated through growth and differentiation factors endogenously produced; second, since the radioprotective effect of endotoxins depends on induction of these factors, as has been confirmed by a lot of data. Furthermore, pharmacological doses of these compounds may be more easily tolerated and more effective than the exogenous immunomodulators or chemical radioprotectors. All this supports the possibility of using them in both prophylaxy and therapy of the consequences of radiation damage (Neta and Oppenheim, 1988). Recently, various endogenous compounds were found to exhibit a protective effect from radiation damage, for example, impure preparations of interferon or colony stimulating factors (Khaitovich et al., 1974; Lvovsky et al., 1977; Urbaschek and Urbaschek, 1983) or precisely defined purified recombinant cytokines (Neta et al., 1986, 1988; Slordal et al., 1989).

In the present work the results indicate that a single and/or systematic treatment with a crude mixture of growth factors present in the cell-free

Abbreviations: TCCM — thymus cell-conditioned medium, cTCCM — five-fold concentrated thymus cell-conditioned medium, i.p. — intraperitoneally, CFU-S — colony forming units in spleen.

filtrate of conditioned medium from rat thymus cells enhances, depending on the dose, the survival of C57BL/6 mice after whole-body gamma irradiation with a dose of 9 Gy.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (Velaz, Praha) weighing about 20 g were used throughout this study. They were held for 10–14 days after delivery to allow for adaptation to the new environment and to recover from any stress of transportation. Mice, 8–10 weeks old, were housed in rodent cages, five to seven animals per cage. They were given LD pellets (Velaz, Praha) and tap water *ad libitum*.

Conditioned medium

Cell-free supernatant of thymus cell-conditioned medium (TCCM) was obtained from Dr. C. Altaner (Cancer Research Institute, Slovak Academy of Sciences, Bratislava). Preparation of conditioned medium was described by Orlik and Altaner (1988). In some experiments, conditioned medium was lyophilized using a Multi Dry (FTS System Inc., USA) freeze dryer. Dry lyophilisate was dissolved in a 5-fold smaller volume of sterile redistilled water immediately before use. Mice were injected intraperitoneally (i.p.) with TCCM in different doses approximately 18 or 8 h before irradiation. Control mice were treated with an equal volume of sterile redistilled water at the same time.

Irradiation

Mice were placed in plexiglass containers and exposed to 9 Gy of total-body gamma rays at a dose rate of 0.3 Gy/min. The Chisostat (Chirana, Czechoslovakia) ^{60}Co source was used for all irradiations.

Survival assay

Studies were initiated in May and concluded in November. Irradiated mice were returned to the animal facility and treated routinely. Survival was monitored daily and was reported as the percentage of animals surviving 30 days postirradiation. Each treatment group within each experiment consisted of 15–30 mice. Experiments were repeated 2 or 3 times.

RESULTS

Female C57BL/6 mice were injected i.p. in a volume of 0.4 ml, 0.8 ml, 1.2 ml 8 h before irradiation. As shown in Fig. 1, pretreatment with TCCM caused dose-dependent radioprotection. No statistically significant differences in

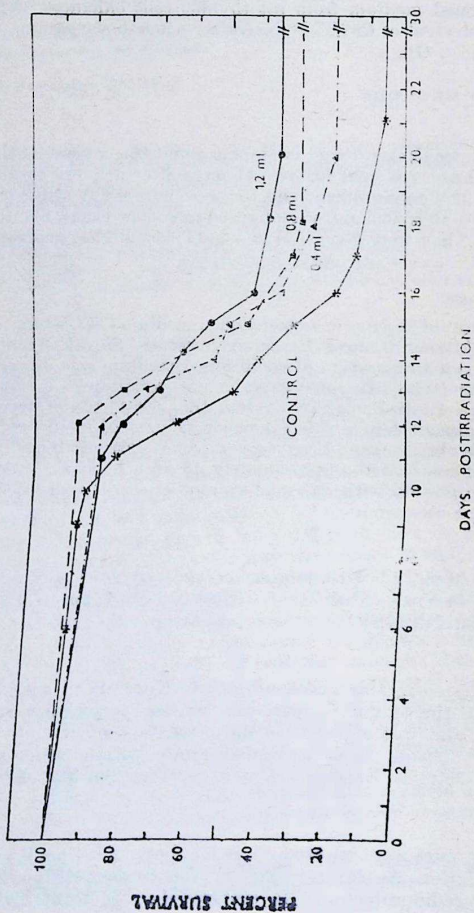


Fig. 1. Radioprotective effects of cell-free supernatant of TCCM on lethally irradiated mice. C57BL/6 mice, 8-10 weeks old, irradiated with a whole-body dose of 9 Gy ($LD_{50/30}$) from a ^{60}Co source. TCCM administered i.p. 8 h before irradiation (see Materials and Methods). Controls (—●—) $n=40$; 0.4 ml of TCCM (—▲—) $n=60$; 0.8 ml of TCCM (—◻—) $n=59$; 1.2 ml of TCCM (—◉—) $n=60$.

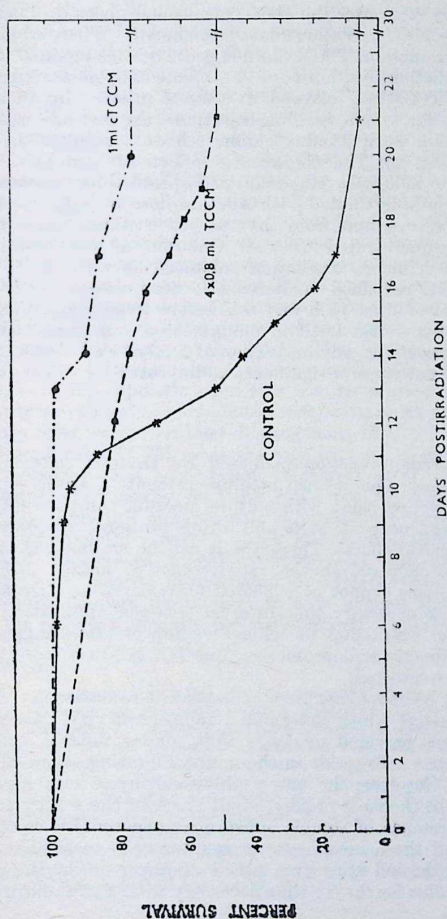


Fig. 2. Radioprotective effect of cell-free supernatant of TCCM on lethally irradiated mice. C57BL/6 mice, 8-10 weeks old, irradiated with a whole-body dose of 9 Gy ($LD_{95/50}$) from a ^{60}Co source. TCCM injected i.p. in 4 doses, once a day in a volume of 0.8 ml (—●—) $n=46$; cTCCM (5-fold concentrated) given in a volume of 1 ml 8 h before irradiation (—○—) $n=56$; (see Materials and Methods).

survival of mice were seen after the above mentioned doses of TCCM given either 18 h or 8 h before irradiation (data not shown). When administered immediately after irradiation, TCCM did not protect mice against the lethal effects of gamma radiation. Furthermore, the effects of systematic treatment with supernatant of TCCM was followed. TCCM was injected i.p. in a volume of 0.8 ml once a day for 4 days, i.e. four injections, the last one being given 8 h before irradiation. Such administration schedule resulted in a 50% survival of mice (Fig. 2). The therapeutic effect of systematic TCCM treatment was not followed. Maximal radioprotection, measured by percent survival of mice irradiated with a lethal dose of gamma radiation, was achieved with supernatant from thymus cell cultures lyophilized and subsequently 5-fold concentrated (cTCCM). One dose of cTCCM injected in a volume of 1 ml 8 h before irradiation enhanced survival to 75%. This percentage of survival remained unaltered also after two doses of cTCCM injected in a volume of 1 ml 18 h and 8 h before irradiation, i.e., the repeated dose did not manifest itself in an increased radioprotective effect (data not shown). In contrast, administration of 1 ml of cTCCM immediately after irradiation did not cause a significant radioprotective effect (data not shown).

DISCUSSION

The mechanism of radioprotection mediated by thymus cell-conditioned medium is not yet clear. Conditioned medium presents a crude mixture of thymus-cell growth factors along with culture medium components such as vitamins, amino acids, inorganic salts and serum proteins, all these having a large scale of biological effects. Therefore, it can be assumed that several mechanisms may be involved in the radioprotective action. Nonspecific action of foreign proteins cannot be excluded (Jurášková and Drážil, 1966; Jurášková, 1971). The necessary time interval between application of conditioned medium and irradiation to achieve radioprotection suggests that TCCM may not be directly radioprotective, but TCCM has to condition the recipients to be radioprotected.

TCCM can protect haemopoietic stem cells against radiation by changing the cell cycle parameters which determined radiosensitivity. Many experimental systems have provided evidence that, under normal conditions, most haemopoietic stem cells reside in the nonproliferating stage of the cell cycle known as G_0 . However, the late S phase cell cycle was reported in numerous studies to be the most radioresistant phase of the cell cycle (Denekamp, 1986). The presence of soluble substances released by cultured rat thymus cells can lead the haemopoietic progenitor cells to S phase of the cell cycle. Therefore, the cell cycle time lasting about 6–20 h (Boggs et al., 1972) may be responsible for the lag time necessary to TCCM radioprotective action.

When mice are exposed to ionizing gamma radiation in a dose range of up to 12 Gy, they will usually succumb, due to haemopoietic failure (bone marrow syndrome). Under radiation conditions used by us haemopoietic cell recovery to normal values is thus considered to be the most important precondition of the survival of mice. Also the remaining amount of dietary supplements of culture medium such as vitamins and amino acids (see Perepelkin, 1965) may play an important role in the recovery process. We are currently investigating this question (results in preparation). However, the influence of the background of individual culture medium constituents used in the TCCM preparation cannot be assessed satisfactorily on the basis of the results obtained after injecting the culture medium *in vivo*. Because of metabolic processes within 48–72 h culture, the initial relative representation of individual constituents of culture medium is changing, so that it does not correspond to the background in TCCM.

As our experiments showed, after TCCM application and subsequent irradiation with a sublethal dose, an increase in the number of bone marrow cells on day 10 following irradiation occurred in comparison with only irradiated C57BL/6 mice (Brezáni et al., 1990). This finding suggests that TCCM application can stimulate bone marrow cells. The accelerative effect on stimulation of myelopoietic stem cells was manifested also in bone marrow transplantation to lethally irradiated recipients, in which myeloid and megakaryocytic cells were identified in exogenous CFU-S (Brezáni et al., 1990). Pretreatment with TCCM, as is the case with many radioprotective agents, induces erythropoiesis acceleration (Macková et al., 1990). Enhancement of erythropoietic recovery in itself could not be responsible for increased survivability, but this enhancement is probably representative of enhanced overall haemopoietic recovery (Ross and Peeke, 1986). Moreover, TCCM application accelerates megakaryocytopoiesis and granulocytopoiesis in spleen and bone marrow in irradiated mice (Macková et al., 1990).

Our results, though preliminary to date, suggest that media conditioned by thymus cells can mediate the radioprotective effect.

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Предварительная обработка мышей средой, кондиционированной тимоцитами, увеличивает выживаемость животных после ионизирующего облучения

П. Федорочко, П. Брезани, Надежда Мацкова

Резюме. Предварительное введение мышам бесклеточного супернатанта — среды, кондиционированной тимоцитами (ТССМ) — в зависимости от дозы защищает их от летального действия ионизирующего облучения. Одноразовое внутрибрюшинное введение ТССМ в объеме 1,2 мл за 8 час. до летального облучения гамма-дозой в 9 Gy ($LD_{95/30}$) спасает 36 % мышей C57BL/6. Четыре внутрибрюшинных дозы ТССМ в объеме 0,8 мл, вводимые раз в день, т.е. в течение 4 дней, защищают 50 % мышей C57BL/6. Интраперитонеальное введение 1 мл сконцентрированной в 5 раз среды (сТССМ) за 8 час. до облучения дозой в 9 Gy сохраняет 75 % мышей. С другой стороны, мы не наблюдали терапевтического действия различных доз этой надосадочной жидкости культуры клеток зубной железы. Наши результаты показывают, что среда, кондиционированная тимоцитами, может опосредовать радиозащитное действие.

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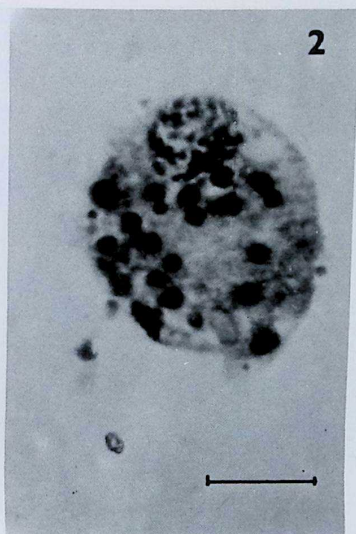


Fig. 2. Coelomocyte with engulfed HEMA particles. May-Grünwald-Giemsa staining, bar = 10 μ m.

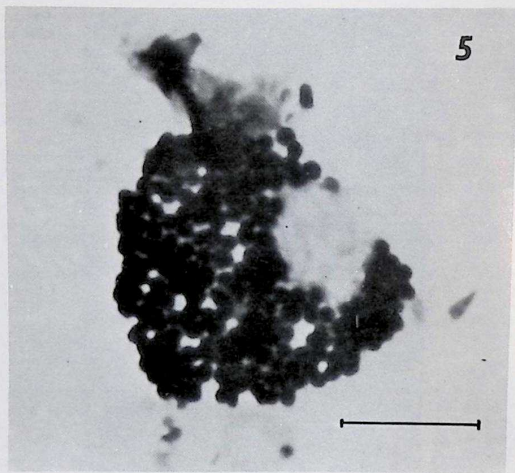


Fig. 5. Coelomocyte with engulfed HEMA particles opsonized in coelomic fluid. May-Grünwald-Giemsa staining, bar = 10 μ m.

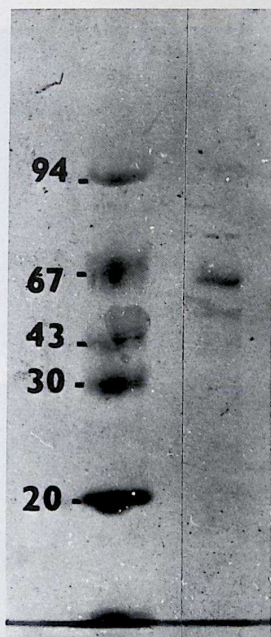


Fig. 6. SDS-PAGE of molecular weight markers (left) and opsonizing proteins (right).



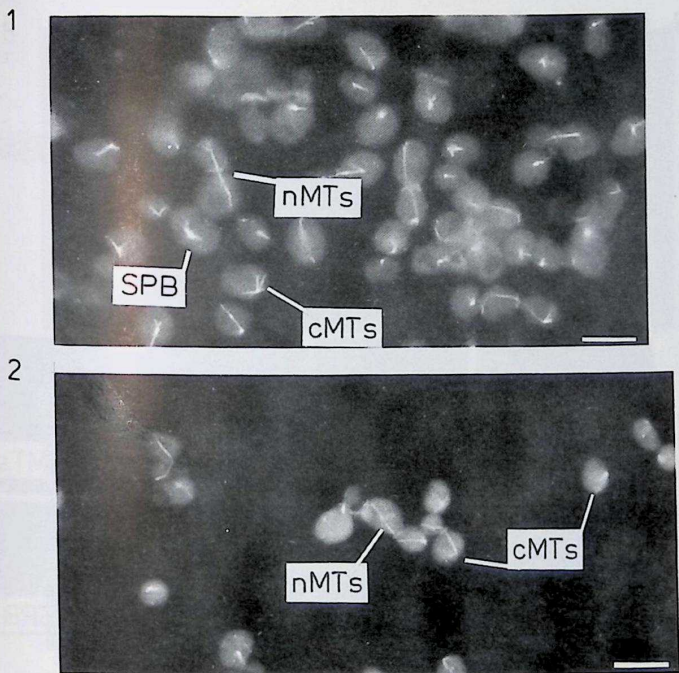


Fig. 1. *S. cerevisiae*, wild type, 1.5 h growth at 24°C. Visible are SPBs, cMTs radiating as fine filaments from SPB, and, in dividing cells, nMTs. Bar, 10 µm.

Fig. 2. *S. cerevisiae*, wild type, 1.5 h growth at 37°C. General pattern of microtubular network as in wild type at 24°C. Cell division is unrestricted. Bar, 10 µm.

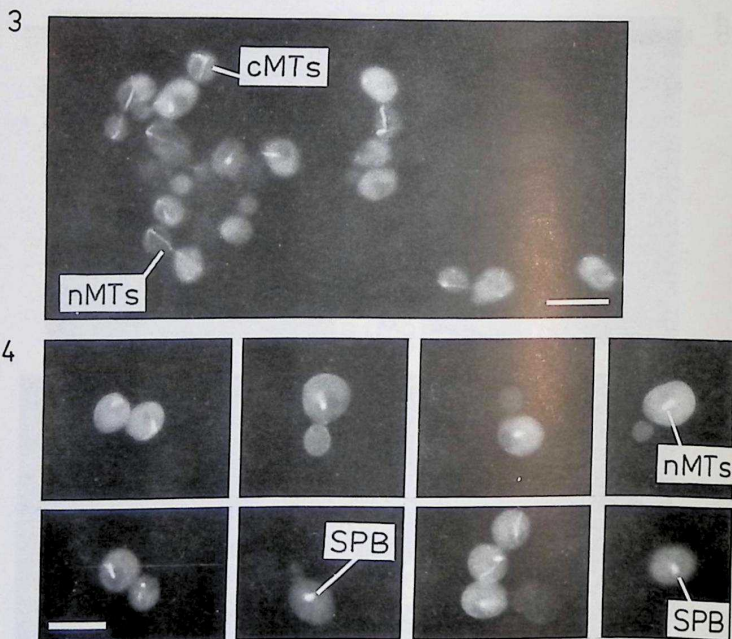


Fig. 3. *S. cerevisiae*, sec 1, 1.5 h growth at 24°C. SPBs, cMTs, nMTs. Bar, 10 μ m.

Fig. 4. *S. cerevisiae*, sec 1, 1.5 h growth at 37°C. cMTs are reduced or absent. Some cells in the S phase have only SPBs and no nMTs. Bar, 10 μ m.

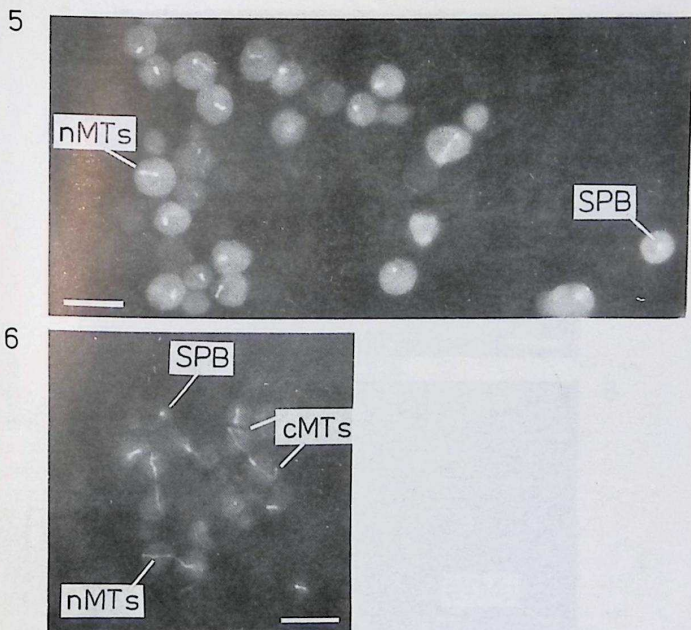


Fig. 5. S. cerevisiae, *sec 1*, 1.5 h growth at 37°C, 2-h postcultivation at 24°C. Mutated phenotype still persisting. Bar, 10 μ m.

Fig. 6. S. cerevisiae, *sec 1*, 1.5 h growth at 37°C, 4-h postcultivation at 24°C. Reversion to original microtubular phenotype. Overall picture corresponds with control. Bar, 10 μ m.

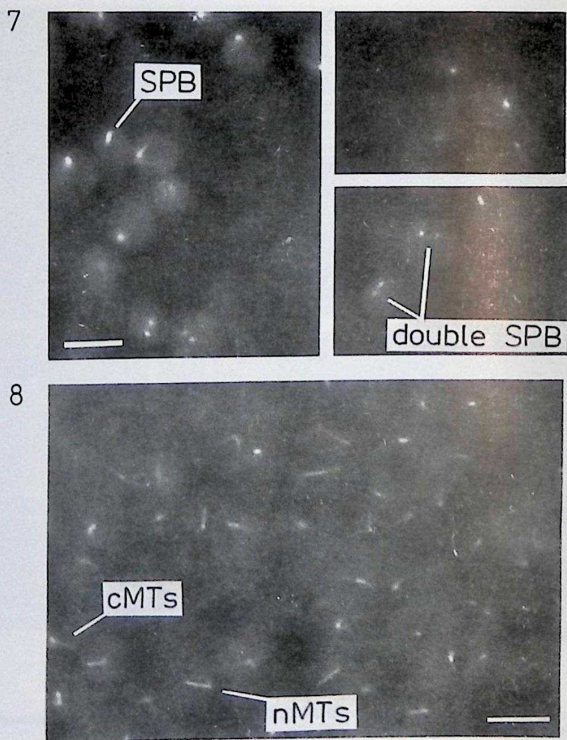


Fig. 7. *Sec 1* protoplasts cultivated 1.5 h at 37°C. Visible are SPBs and duplicate SPBs. Bar, 10 μ m.

Fig. 8. *Sec 1* protoplasts cultivated 1.5 h at 24°C. SPBs, newly developing cMTs. Bar, 10 μ m.

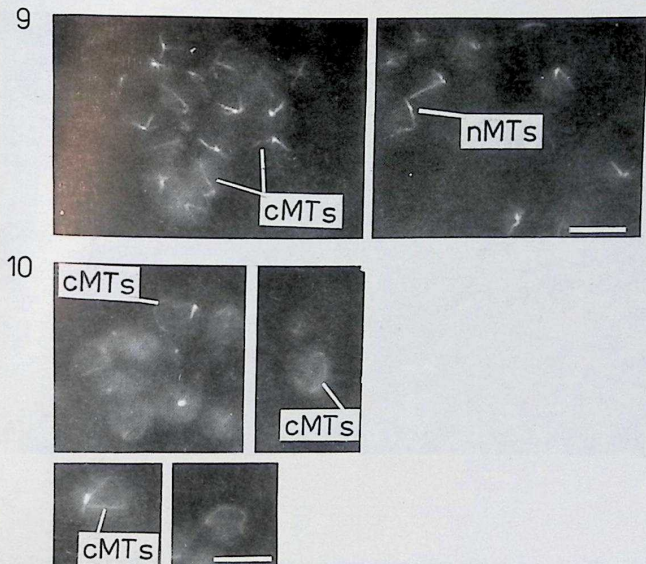


Fig. 9. *S. cerevisiae*, *sec 7*, 1.5 h growth at 24°C. SPBs, cMTs, nMTs. Bar, 10 μ m.

Fig. 10. *S. cerevisiae*, *sec 7*, 1.5 h growth at 37°C. MTs are unimpaired, cMTs run as if along the periphery of cells. Cytokinesis is not visible. Bar, 10 μ m.

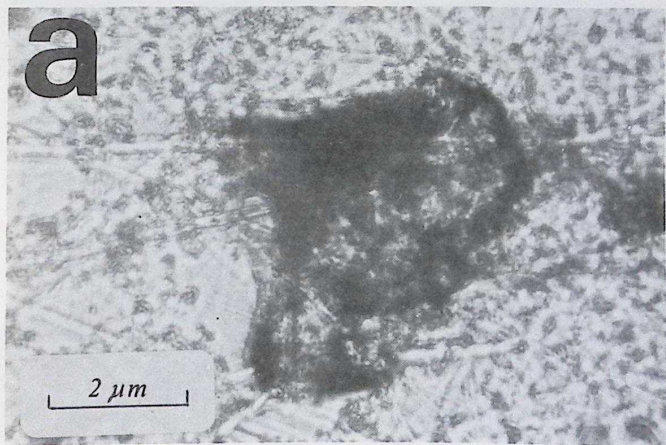


Fig. 3. Protein aggregates from human lens in a light microscope ($625\times$): a — for $M = 4$, b — for $M = 5$, c — for $M = 6$.

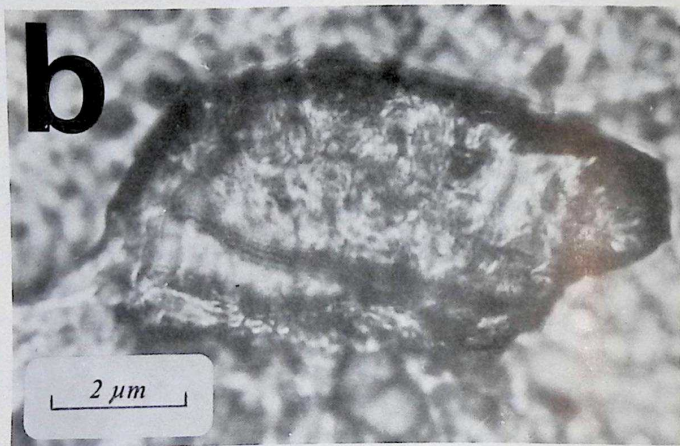


Fig. 3b.

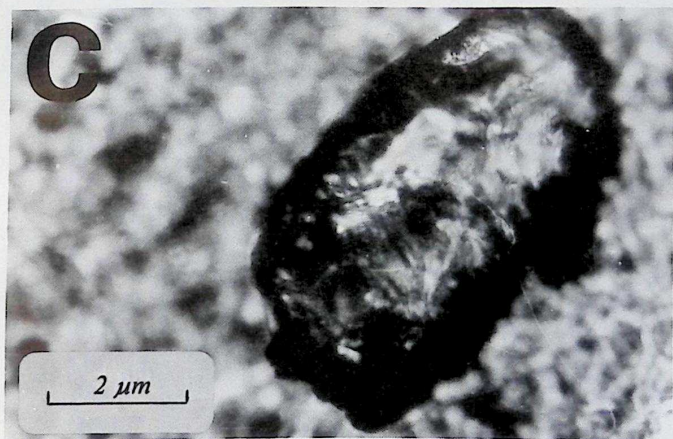


Fig. 3c.

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