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A gelatin sponge model for studying tumor growth: quantitation of tumor cells and leukocytes in the CHO tumor[1]

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Summary. A gelatin sponge model system for tumor cell inoculation and retrieval of tumor-associated leukocytes is described. Gelatin sponges pre-implanted in nude mice harboring tumorigenic Chinese hamster ovary cells (line CHO) were examined at 2 and 11 days after injection of tumor cells for tumor cell content and leukocyte accumulation after digesting the sponge matrix in collagenase solution.

The data indicate a progressive influx of host cells consisting primarily of macrophages, neutrophils and lymphocytes. The total number of viable tumor cells as well as the fraction of surviving tumor cells with clonogenic potential also increased with tumor age. Blank sponges not harboring tumor cells elicited an inflammatory response in the animals which did not change appreciably with length of sponge residence. However, when the sponges were harboring tumor cells, the accumulation of host leukocytes far exceeded that which occurred in blank sponges. This observation suggests a host response directed toward the tumor which is absent in animals bearing blank sponges. Apart from providing anchorage for injected cells, the gelatin sponge, by virtue of its digestibility in collagenase, makes possible the easy retrieval and precise quantitation of tumor-associated host cells.

Key words. Tumor cell inoculation; tumor-associated leukocytes; gelatin sponge; host response; host cell influx.

Introduction

Several reports have demonstrated anti-tumor immune responses in tumor bearing animals. The host response is generally characterized by the accumulation at the tumor site of host elements, some of which are tumoricidal (reviewed by Evans[3] and Kreider et al.[4]). Hayry and coworkers[5,6] utilized urethane sponge matrices that were recoverable and from which viable and functional allograft infiltrating host cells could be obtained by gentle compression. This model has also been used in the characterization of T-cell mediated antitumor immune responses[7]. We have employed[8–10] a pre-implanted gelatin sponge model that provides anchorage for the injected cells and makes possible the retrieval and quantitation of tumor cells and infiltrating host cells after collagenase digestion. While earlier reports[5–7,11] have documented the host immune response to sponge allografts and tumor-containing sponges, the potential foreign body reaction that can be induced by these sponges has been often assumed to be inconsequential. Recently, we showed that blank sponges implanted in nude mice elicit a classic inflammatory response which is characterized by an early non-sustaining influx of granulocytes followed closely by a persistent macrophage accumulation[10]. A question that remains to be resolved is whether tumor-containing sponges elicit a host response which is different from the foreign body reaction induced by blank sponges.

The study described here represents a progression of ongoing research in our laboratory which has documented the phenotypic[9] and chromosomal[12] changes occurring in Chinese hamster lineages during tumorigenesis. The nude mouse chosen for these studies is immune-deficient and therefore permits the study of tumor progression of these lineages in vivo with minimal immunological host intervention. This study was designed to quantify the host cells recruited into the matrix of a gelatin sponge that had been inoculated with tumor cells and to compare the extent of host cell infiltration with that occurring in sponges lacking tumor cells (blank sponges). Concomitantly, the fate of the injected tumor cells was determined.

Host cells infiltrating gelatin sponges pre-implanted in nude mice and injected with tumorigenic Chinese hamster cells (line CHO) were analyzed at 2 days and 11 days after the injection of tumor cells. The time points chosen for analyses represent the early (non-palpable) and the later (palpable) stages of tumor growth. We present evidence that demonstrates the increase in the numbers of tumor-associated macrophages, lymphocytes and granulocytes with tumor age. Blank sponges also cause an initial accumulation of these cell types, however, in smaller numbers that changes little with time of sponge residence in the animal. The results also indicate that the number of tumor cells with clonogenic potential represent only a fraction of the total tumor cells present in the tumor mass.

Materials and methods

Animals. Eight- to twelve-week-old female Balb/c nude mice which originated from breeding pairs (nu/nu males X nu/+ females) obtained from ARS/Sprague-Dawley, Madison, WI, were used in these studies. Conditions under which the animals were maintained and bred have been previously described[8].

Tumorigenic cell line. Chinese hamster cells (line CHO), that were HPRT$^-$ and 6-thioguanine resistant were used. The cells were maintained in culture as monolayers in Ham's F-10 medium containing 5 mg/ml 6-thioguanine (Sigma, St. Louis, MO) and supplemented with 10% dialyzed fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin.

Preparation of mouse CHO antiserum. Each of 30 female Balb/c mice were injected i.p. with 10^7 viable CHO cells at monthly intervals for a total of 5 doses. 14 days after the final injection the mice were lightly anesthesized with ether and exsanguinated. Sera from all animals were pooled, aliquoted and frozen at $-20\,^\circ C$.

Sponge implantation and injection of tumorigenic cells. Sterile, cut, gelatin sponges (Spongostan, Ferrosan, Denmark) each measuring approximately 17 × 18 × 10 mm were surgically implanted s.c. to the upper back of pentobarbital anesthesized mice as previously described[10]. Approximately 10^5 exponentially growing CHO cells in 0.1 ml saline were injected into sponges 2 days after sponge implantation.

Sponge retrieval and disaggregation. Tumor sponges and blank sponges were surgically removed at selected intervals from animals sacrificed by CO_2 inhalation. Sponge disaggregation was accomplished by a modification of the method described by Kerkof[13] for thyroid gland dissociation. The prewarmed enzyme cocktail consisted of 25 ml saline G (composition in g/l: glucose 1.1 g, NaCl 8.0 g, KCl 0.4 g, $Na_2HPO_4 \cdot 7\,H_2O$ 0.29 g, KH_2PO_4 0.15 g, phenol red 0.005 g, $MgSO_4 \cdot 7\,H_2O$ 0.154 g, $CaCl_2 \cdot 2\,H_2O$ 0.016 g) containing 50 mg collagenase (Worthington, Biochemicals, Freehold, NJ) and 500 mg bovine serum albumin (Fraction V, Sigma, St. Louis, MO). Sponge explants (pooled from 3 mice) placed in a 10-ml beaker were minced with a pair of scissors. Minced sponges (tumor or blank) were immediately resuspended in 5 ml of enzyme mixture and transferred with a 10-ml pipette into 25-ml spinner culture flasks (Bellco, Vineland, NJ). Enzyme solution was added to bring the total volume to 25 ml. Spinner culture flasks were incubated at 37 °C for 1.5–2 h with constant stirring. The resulting cell suspension was filtered through 380-μm and 140-μm wire screens in that order. All manipulations were performed under sterile conditions.

Determination of cell yield and viability. The viability and yield of dispersed cells was determined by counting the cells in a hemocytometer under mercury lamp illumination after staining with propidium iodide (PI) and acridine orange (AO)[14]. The dyes were excited (blue excitation) using an exciter filter (BP 450–490), chromatic beam splitter (FT 510) and a barrier filter (LP 520). 0.1 ml of cell suspension was added to 10 μl propidium iodide (200 μg/ml in PBS) and 100 μl acridine orange (10 μg/ml in PBS). Viable cells are stained with acridine orange (green nuclear fluorescence) and dead cells are stained with propidium iodide (red nuclear fluorescence). In this technique, only nucleated cells are stained; red blood cells lacking a nucleus are not stained and, therefore, do not interfere with the cell count. For these studies the mean cell viability of blank or tumor sponge derived cells was at least 94%.

Antibody staining of cell suspensions. Cells obtained from blank or tumor-containing sponges were centrifuged (200 × g, 10 min) and resuspended to 10^7 cells/ml in dilution fluid [a-MEM containing 2 N morpholino propane sulfonic acid (a-MOPS) supplemented with 3% fetal bovine serum (FBS) and containing 0.1% sodium azide]. Prior to staining, all antibody reagents were centrifuged at 60,000 × g for 15 min in a Beckman airfuge to remove aggregates. All steps of the staining procedure were carried out on ice. The cell suspension (100 μl) was added to an equal volume of a 1:25 dilution of anti-CHO immune serum or control normal mouse serum (final antibody dilution was 1:50). Antibody labeling was allowed to proceed for 45 min on ice and then terminated by two washes (200 × g, 4 °C) in dilution fluid. In order to identify the labeled cells, fluoresceinated $(Fab)_2'$, fragments of goat-anti-mouse (FGAM) kappa and heavy chain-specific immunoglobulin (TAGO, Inc) was used as a second reagent. The affinity-purified FGAM was used at a dilution of 1:3. The cells were stained for 15 min in FGAM and then washed once in dilution fluid. This was followed by lysis of red blood cells in 0.83% ammonium chloride for 1 min and an additional wash. Cells were then resuspended in 2 ml of saline supplemented with 2.5% FBS and 0.1% sodium azide for flow analysis. As a check for specific binding of the immune serum, exponentially growing cultured CHO cells were similarly stained with the anti-CHO serum or normal mouse serum. Immediately prior to flow analysis, cells were stained for 5 min with propidium iodide (10 μl of 200 μg/ml stock solution) to exclude dead cells stained with PI from the analysis[14].

Flow cytometric analysis. Flow analysis of fluorescently labeled cells was performed on a Los Alamos designed flow cytometer[15]. Fluorescein isothiocyanate (FITC) and propidium iodide (PI) were excited at 488 nm with an argon-ion laser. The appropriate combination of filters allowed the measurement of green fluorescence from FITC-stained cells at 515–545 nm and the red nuclear fluorescence of PI-stained cells (dead cells) at 610 nm. In addition to the fluorescence measurements, physical parameters of Coulter volume and forward angle light scatter were also measured. A PDP-11/23 computer was used for data collection and storage. Reprocessing of the multiparameter data using the LACEL program[16] allowed the resolution of viable CHO cells stained with the species-specific anti-CHO serum.

Phagocytosis. Phagocytic activity is routinely used to functionally identify adherent macrophages. Phagocytosis of heat-killed baker's yeast by adherent cells in the presence of complement was measured by a previously described method[17]. Briefly, 50 μl of yeast suspension (1.4×10^8/ml) and 0.3 ml guinea pig complement (1:9 dilution in saline) were added to a monolayer of adherent cells. After 30 min incubation at 37 °C, the monolayer was rinsed with warm phosphate buffered saline (PBS), counterstained with 0.4% methyl green for 10 min and mounted onto microscope slides using permount. 200 cells were counted under oil immersion, and phagocytosis

was expressed as the percent of cells ingesting yeast particles.

Histological identification of neutrophils and lymphocytes. Lymphocytes and neutrophils were identified after histological examination of Wright's stained cytocentrifuge cell preparations. Methanol fixed slides were stained with Wright's stain (Camco Quick Stain, American Scientific Products) for 2 min, rinsed in distilled water, and air dried. 200 cells were examined in random sequential fields under oil immersion.

Clonogenic cell survival assays. In order to determine the number of surviving tumor cells with clonogenic potential, tumor-containing sponges were aseptically removed from sacrificed animals and disaggregated. Following tumor dissociation, the cell suspension was counted in a hemocytometer and various 10-fold dilutions (10^6/ml to 10^2/ml) of this suspension was plated in F-10 medium containing 5 mg/ml of 6-thioguanine. This selective medium supports the proliferation only of the thioguanine-resistant CHO tumor cells in the cell suspension. Culture plates were incubated for 8–10 days at 37 °C in a 5% CO_2 atmosphere. Colonies were rinsed carefully in saline, and fixed in absolute methanol for 10 min. Fixed cells were rinsed free of methanol and stained for 10 min in aqueous May Grünwald-Giemsa stain. Colonies consisting of at least 50 cells were counted under an inverted stage microscope.

Results

Cell recovery from sponges. The host response to pre-implanted sponges was determined on day 2 and day 11 from 3 pooled sponges (blank or tumor) obtained from 3 mice for each experiment. The sponges were digested in collagenase and the total cells recovered were determined after counting in a hemocytometer. Figure 1 shows data obtained from these enumerations. By day 2 post-inoculation of 10^5 CHO cells (4 days post-implantation of sponges), there was an accumulation of host cells (as well as tumor cells) as evidenced by the large number of cells recovered from the sponges. Sham-injected blank sponges also contained a similar number of cells. The total number of cells in blank sponges remained relatively unchanged by day-11. However, day-11 sponges harboring early CHO tumors contained at least 6 times as many cells as blank sponges.

Tumor cell content of CHO tumors. In order to determine the contribution of tumor cells to the total number of cells recovered from tumor sponges, CHO cells were quantitated by flow cytometric (FCM) analysis of disaggregated tumor suspensions labeled with indirectly fluoresceinated, anti-CHO immune serum. Figure 2 shows the fluorescence distributions of cultured CHO cells and 2-d and 11-d cell suspensions derived from tumor sponges. Normal mouse serum used as an indicator of non-specific binding displayed negligible staining of cultured CHO cells or of cells recovered from 2-d tumor sponges (fig. 2). Day-11 tumor sponge-derived cells, however, displayed a measurable amount of non-specific staining with normal mouse serum. In such cases, the fraction of cells staining non-specifically with the fluoresceinated normal mouse serum was determined and used as a baseline above which the fluorescence due to the

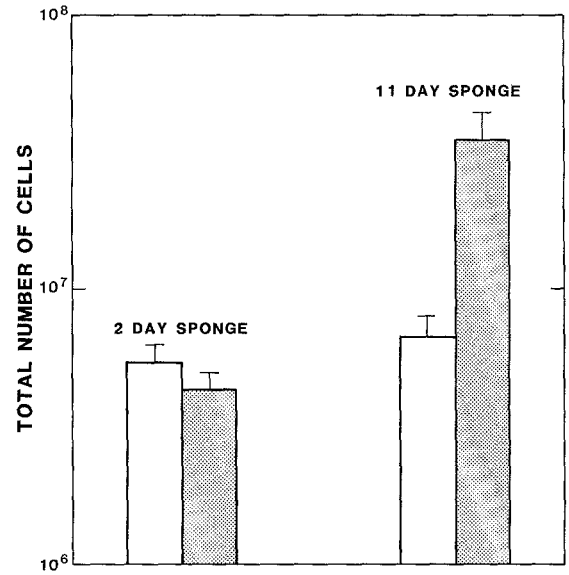

Figure 1. Total number of cells recovered from blank (□) or tumor (▒) sponges at 2-d or 11-d post inoculation of CHO cells. Each bar represents the mean ± SEM of 5 experiments using 3 pooled sponges for each time point.

binding of the species-specific immune anti-CHO serum was calculated[18]. There was also no significant binding of immune anti-CHO serum to 2-d or 11-d blank sponge-derived cells (data not shown). The anti-CHO serum was species-specific and stained at least 97% of cultured CHO cells analyzed (fig. 2). In cell suspensions obtained from tumor sponges stained with anti-CHO immune serum, the percentage of cells stained was higher in the 11-d (19.95%) than in the 2-d (1.37%) tumor sponges.

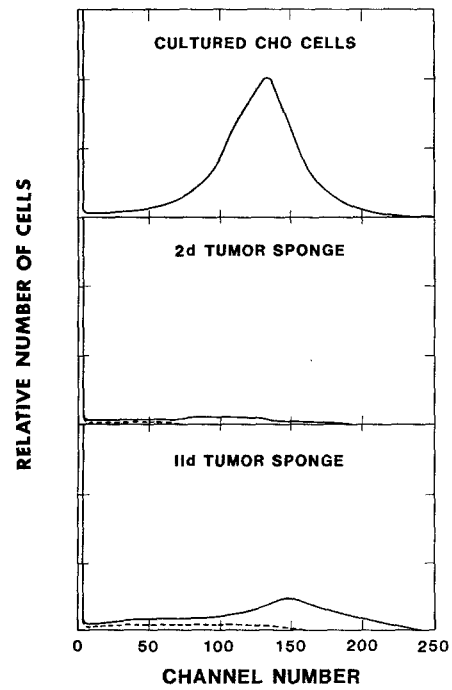

Figure 2. Fluorescence distributions of cultured CHO cells and tumor sponge suspensions labeled with indirectly fluoresceinated normal mouse serum (--) or anti-CHO immune serum (–). The green fluorescence intensity is proportional to the channel number which is displayed on the x-axis. 10,000 cells were analyzed to generate the fluorescence histograms.

Table 1. Clonogenic potential of tumor cells obtained from 2-d and 11-d sponges

	2-Day	11-Day
Total tumor cells per sponge[a]	8.67×10^4	11.06×10^6
Total clonogenic tumor cells per sponge[b]	2.74×10^4 (31.6%)	7.94×10^6 (71.8%)

[a] The total number of tumor cells was determined by flow cytometric analysis of samples stained with anti-CHO immune serum. Values represent the mean cell number determined from 2 experiments. Sponges from 3 mice were pooled for each experiment and for each time point. [b] Values represent clonogenic cells after correction of the plating efficiency, in selective medium, of cultured CHO cells (PE = 0.82). Numbers in parentheses represent percentages of viable tumor cells with clonogenic potential.

Table 2. Host cells infiltrating sponges[a]

	Cells per sponge ($\times 10^6$)			
	2-Day		11-Day	
	Blank	Tumor	Blank	Tumor
Monocyte/ macrophage	3.20 ± .52	2.26 ± .39	3.39 ± .28	14.18 ± 3.9
Neutrophils	.85 ± .57	.52 ± .22	.72 ± .53	3.48 ± .48
Lymphocytes	.07 ± .007	.10 ± .03	.05 ± .005	0.27 ± .05

[a] Values represent the mean ± SEM of 3 experiments. Macrophages were identified after ingestion of complement coated yeast particles. Neutrophils and lymphocytes were differentially identified on Wright's stained cytocentrifuge preparations.

Clonogenicity survival assays. The number of tumor cells in the tumor sponge that retained proliferative potential was determined by plating tumor suspensions in growth medium containing 6-thioguanine. Concomitantly, tumor cell number was determined from FCM analysis of cell suspensions stained with CHO-specific immune serum. The results shown in table 1 indicate that day-11 tumor sponges contain a greater number of tumor cells with clonogenic potential than day-2 tumor sponges. In both cases, however, the clonogenic populations represent only a fraction of the total number of recovered viable tumor cells. Two-day tumor sponges contained a smaller clonogenic fraction of tumor cells (32%) than 11-d tumor sponges (72%).

Identification of host cells in sponges. The number of macrophages infiltrating the sponges was determined after the ingestion of yeast particles by plastic adherent cells from previously disaggregated sponges. Neutrophils and lymphocytes were identified histologically in Wright's stained cytocentrifuged preparations. Table 2 shows the absolute numbers of host cells infiltrating blank and tumor sponges. Of the cell types examined, macrophages constituted the greatest number of host cells, followed in order by neutrophils and lymphocytes. The number of host cells identified in blank sponges remained relatively unchanged with time. This contrasts with tumor-containing sponges which manifest a substantial increase in macrophages with tumor age.

Figure 3 is the result of the transformation of the actual numbers of macrophages, neutrophils, lymphocytes and tumor cells to give a distribution which shows the proportions of these cells relative to one another. Macrophages made up the largest fraction of cells recovered from blank or tumor sponges. Tumor cells constituted only a small fraction of the total cells recovered from day-2 sponges; this fraction had increased considerably by day-11 and approached that of macrophages.

Discussion

The data from these studies indicate a host response to tumorigenic Chinese hamster cells (line CHO) injected in pre-implanted gelatin sponges that is manifested by the enhanced influx of macrophages, lymphocytes and neutrophils into the inoculation site. Two-day tumor sponges (4 days post-implantation of sponge) caused the recruitment of the host cells in numbers comparable to 2-d blank sponges lacking CHO cells. By day 11, however, the macrophage, lymphocyte and neutrophil content of tumor sponges had increased while blank sponges showed little change from day 2. The quantitation of tumorigenic CHO cells stained with anti-CHO immune serum revealed an early decrease in the number of recovered viable tumor cells (8.67×10^4) below the initial inoculum of 1×10^5 cells at day 2 post-injection. This decrease in tumor cell number by day 2 post-injection is very unlikely to be due to a specific anti-tumor response since a similar loss in cell numbers has been reported for early passage, non-tumorigenic Chinese hamster cells implanted in nude mice[10] as well as for normal rat thyroid or mammary cells injected into syngeneic recipients[19,20]. The fraction of viable tumor cells in 2-d sponges with clonogenic potential on selective medium was also considerably diminished (32%) compared with 11-d tumor sponges where an increase in tumor cell clonogenicity (72%) was correspondingly accompanied by an 11-fold increase in the total number of viable tumor cells recovered.

The accumulation of leukocytes in the blank sponges is indicative of a classic inflammatory response elicited by the gelatin sponge matrix that we described earlier[10]. The recruitment of macrophages, neutrophils and lym-

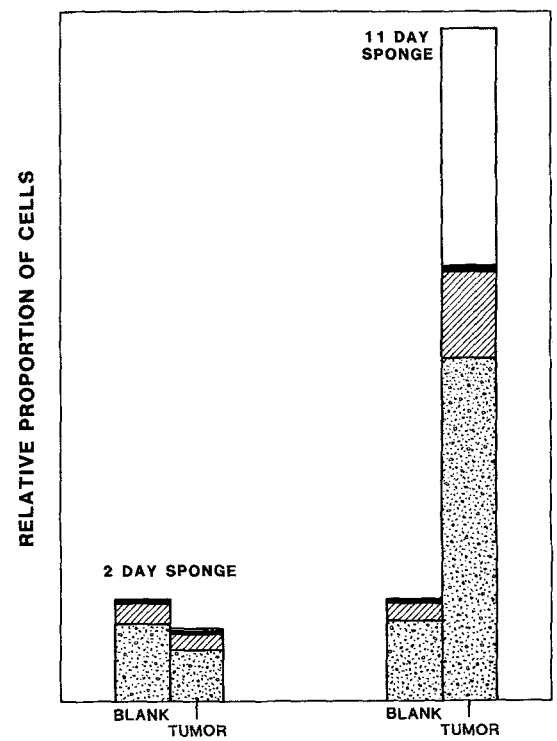

Figure 3. Schematic representation of macrophage (▨), neutrophil (▨), lymphocyte (■) and tumor cell (□) content of 2-d and 11-d sponges.

phocytes in greater numbers in 11-d tumor sponges may be the result of a continuing inflammatory response similar to that occurring in blank sponges. Alternatively host cell recruitment into the tumor sponge may be the result of a specific host recognitive event to the transplanted tumorigenic cells[7].

These observations are in agreement with the earlier findings of Hayry and Roberts[5] who demonstrated, by histological examination, a prominent inflammatory response to tumor-bearing implanted polyurethane sponges that vastly exceeded that which occurred in blank sponges. Our study employing pre-implanted gelatin sponge matrices has identified and quantitatively determined the numbers of host and tumor cells in a tumor system following the digestion of the sponge matrix in collagenase. The earlier documentation in our laboratory of conspicuous vascularization of whole retrieved blank gelatin sponges pre-implanted in nude mice[10], we believe, justifies complete solubilization of the gelatin matrix to ensure total cell recovery. In earlier studies[5-7, 11], employing polyurethane or viscous cellulose sponges, gentle compression of recovered sponges was thought to be sufficient for complete cell retrieval. This method was considered adequate since histological examination revealed the localization of granulation tissue only to the superficial layers of the sponge[5].

The often stated advantage of the 'compression' technique is that the recovered cells are spared any trauma due to enzyme digestion. Collagenese digestion is a routinely used method of tumor disaggregation and has been demonstrated not to affect the expression of surface immunoglobulin (SIg) and brain associated thymus antigen (BATA) on B and T lymphocytes respectively[21].

More recently[14] it has been reported that limited exposure to collagenase does not alter the expression of Fcγ receptors on 5-day cultured murine bone marrow macrophages. We have also shown in our laboratory (unpublished data) that collagenase digestion to recover CHO cells cultured in vitro in gelatin sponges neither diminishes the recovery nor the plating efficiency of these cells. This study has quantitated some of the host cells infiltrating a tumor sponge as well as determined the fate and clonogenic capacity of surviving tumor cells. By providing anchorage for injected cells as well as acting as a 'trap' for infiltrating host cells with potential effector functions, the pre-implanted sponge model furnishes a retrievable arena for studying in vivo host-tumor cell interactions. The delineation of host cells and the fate of tumor cells from the tumor mass can be studied in this model and will allow future studies in which the injection of tumor or leukocyte-derived factors into pre-implanted sponges would make it possible to elucidate the mechanism(s) of host cell recruitment into a tumor.

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Short Communications

Physiological role of apyrene spermatozoa of *Bombyx mori*

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Summary. Observation by electron and phase-contrast microscopy demonstrated that in *Bombyx mori* the dissociation of eupyrene bundles, apparently through digestion of the prostatic secretion or an endopeptidase, was promoted by the vigorously flagellating movement of apyrene spermatozoa in the spermatophore.

Key words. Apyrene spermatozoa; eupyrene spermatozoa; spermatophore; sperm maturation; glandula prostatica; endopeptidase; *Bombyx mori.*