MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. IV, pp. 97-107, March 1971

Studies on a Specific Structure in Differentiating Slime Mold Cells

By

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(Received December 3, 1970)

ABSTRACT Formation and fate of a unique vacuole, which was specifically present in the prespore cell of the cellular slime mold *D. discoideum*, were studied in relation to the spore-specific antigens contained in the vacuole.

The vacuoles (PSV) were excreted from the prespore cells during their cytodifferentiation into the spores, and their contents constituted the spore coat. Judging from its size and distribution as well as its behavior during spore formation, the PSV was considered to be identical with a cytoplasmic granule (of the prespore cell) which reacted specifically with a heteroplastic antispore serum. The antispore serum absorbed with the acid mucopolysaccharide(s) of the fruiting body stained neither the cytoplasmic granules in the prespore cell nor the surface of the spore, indicating that a major component of the spore-specific antigens is the acid mucopolysaccharide(s). Galactose which was a major component of the acid mucopolysaccharide(s) was specifically incorporated into the prespore cells, but not into the prestalk cells of the slug. This showed that synthesis of the acid mucopolysaccharide(s) is limited to the prespore cells.

Introduction

The life cycle of the cellular slime molds includes the following steps : (1) Germination of spores into vegetative amoebae. (2) Growth and cell multiplication by feeding on a suitable species of bacteria. (3) Cessation of growth by exhausting external bacteria. (4) Aggregation of amoebae into an organized assembly (aggregation stage). (5) Formation of a papilla-like cell mass from the aggregate (papillate stage). (6) Development of the cell mass into a migrating pseudoplasmodium of a slug shape (migration stage). (7) Formation of a fruiting body composed of a mass of spores and a supporting cellular stalk

(culmination stage).

During culmination, the anterior part of the slug differentiates into stalk cells, while the posterior part differentiates into spores. It has been demonstrated by an immunohistochemical study using fluorescein conjugated antispore sera that spore-specific antigens are present in cytoplasmic granules of the prespore cells, but that none of them was found in the prestalk cells (Takeuchi, 1963). An electronmicroscopic study has also revealed that a unique vacuole named PSV has been found specifically in the cytoplasm of the prespore cells, but not in the prestalk cells (Maeda and Takeuchi, 1969 ; Hohl and Hamamoto, 1969).

It is the purpose of the present work to investigate formation and fate of the PSV during the course of development of this organism and relation of the vacuole to the spore-specific antigens.

Materials and Methods

Organisms and cultivation

Dictyostelium discoideum, strain NC-4 was the organism mainly used. *D. mucoroides*, No. 11 was used for preparation of antispore sera. They were grown on a standard nutrient agar medium with *Escherichia coli* (Bonner, 1947). Amoebae were harvested from growth plates at the stationary growth phase and washed free of bacteria by repeated centrifugations in chilled standard salt solution (Bonner, 1947). Washed amoebae were incubated on non-nutrient agar at 21°C, and cell masses were selected at the proper stages of their development.

Electronmicroscopic procedures

Cell masses at the various stages of development were fixed in 1% OsO_4, dehydrated, and embedded in Epoxy resin. The detailed procedures for these steps were the same as described previously (Maeda and Takeuchi, 1969). Sections (600-800 Å thick) double-stained with uranyl acetate and lead citrate were observed with a Hitachi HU-11D.

Preparation of fluorescein conjugated antispore sera.

Antisera were prepared against spores of *D. mucoroides*, No. 11, according to the methods as described by Takeuchi (1963). The obtained antisera were conjugated with fluorescein isothiocyanate (Baltimore Biochemicals), according to the method described by Marshall *et al.* (1958). The conjugated antiserum was purified by fractionation through a DEAE-cellulose column, as described by Riggs *et al.* (1961). Pre-injection sera were conjugated and fractionated in the same way as for the antiserum.

Preparation of the antisera absorbed with the acid mucopolysaccharide(s).

An acid mucopolysaccharide(s) was isolated from mature fruiting bodies of *D. discoideum*, according to the method described by White and Sussman (1963). The purified acid mucopolysaccharide(s) (8 mg) was added to a fluorescein conjugated antispore serum (0.5 ml). The mixture was incubated at 37°C for 1.5 hours, and was kept overnight in a refrigerator. It was then centrifuged at 30,000 g for 30 min. in cold, and the resulting supernatant was used as an

absorbed antiserum.

Immunohistochemical methods

Slugs of *D. discoideum* were sandwiched between non-nutrient agar and a coverslip and were incubated in a moisture chamber, at 21°C for 2 hours, before fixation. Spores were smeared on a coverslip and dried in the air. Fixation of the cells was done in ice-cold methyl alcohol, then washed in three changes of 10 mM phosphate buffered saline, pH 7.0. These preparations were incubated in a fluorescein conjugated serum for 20 min., at room temperature. They were then washed in three changes of the buffered saline, followed by mounting in a mixture of 9 parts of glycerin and 1 part of the buffered saline. The preparations were examined in a dark field with a Nikon Fluorescence Microscope equipped with a Toshiba SH 200 light source. A BV exciter with a Nikon blue barrier filter was used.

Autoradiographic methods

Washed amoebae of *D. discoideum* were placed on a millipore filter (pore size, 0.45 μ) resting on a absorbent pad saturated with 50 μCi/ml of ^{3}H-galactose (specific activity, 2.9 Ci/mM) and were incubated at 21°C in a petri dish. After the incubation of 24 hours, slugs were picked up from the filter and were fixed in ice-cold methyl alcohol. After washing, the fixed slugs were squashed and coated with a fluid emulsion Sakura NR-M2. The coating was done about 3 feet away from a Wratten safelight No.2 in a completely light-proof dark room. The coated material was stored in a black plastic box at 4°C for 10 days.

The exposed film was developed in a Sakura Conidol X at 20°C for 4 min. and was fixed in a Sakura Conifix at 20°C for 10 min. This was then washed in running water for 20 min. and was dehydrated in 95% alcohol and in two changes of absolute alcohol. The autoradiographs were mounted by a coverslip with Canada balsam.

Results

1. *A structure (PSV) specific to the prespore cells.*

In the prespore cells of the slug there were a considerable number of specific vacuoles of 0.5-0.7 μ diameter (Maeda and Takeuchi, 1969). The unit membrane of the vacuole was lined with an electron dense membraneous structure of ca. 200 Å thick which enclosed an aggregate of filaments of ca. 50 Å diameter (Fig. 1). The vacuole was characteristic of the prespore cell and was never found either in the prestalk cell of the slug or in the amoeba at the aggregation stage. Therefore, this vacuole was named a prespore-specific vacuole (PSV). The PSV was found to be a sole structure that showed discrete localization between the prespore and prestalk cells. A structure similar to the PSV was independently found and was named PV by Hohl and Hamamoto (1969) in their recent paper.

2. *Excretion of the PSV and spore coat formation.*

The PSV was found not only in the prespore cells of the slug but also in

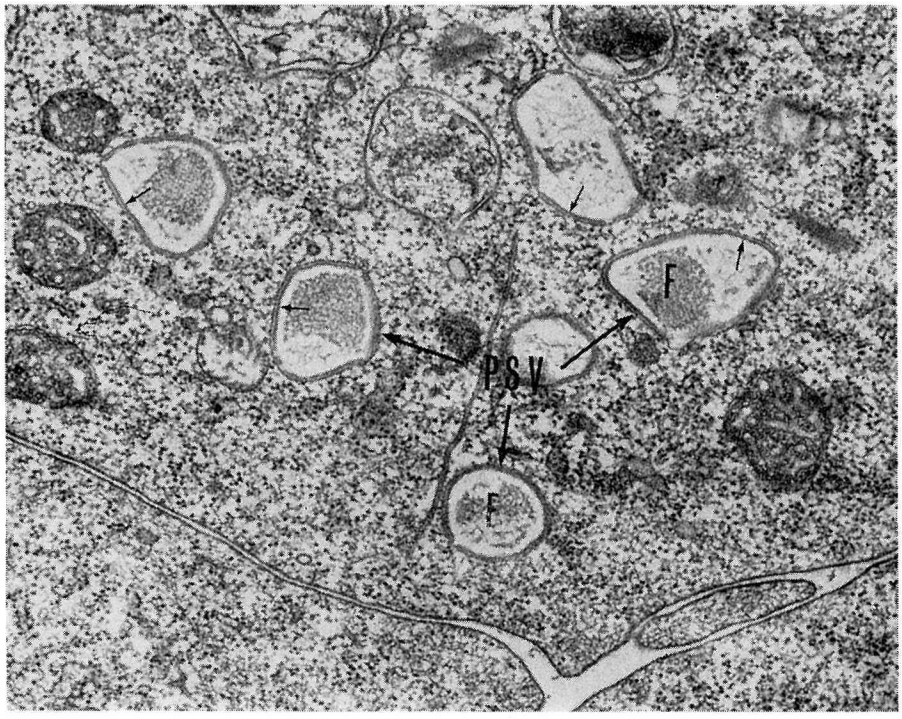

Fig. 1. The prespore-specific vacuoles (PSV) in a prespore cell of *D. discoideum*. The unit membrane of the vacuole is lined by a membraneous structure (arrows) of high electron density, which encloses an aggregate of filaments (F). X 26,700.

those of the culminating cell mass. This vacuole, however, was never found in the cytoplasm of mature spores which had completed coat formation (Fig. 2). These facts suggest that the PSV is excreted from the cytoplasm during formation of mature spores. In order to examine this possibility, prespore cells of the culminating cell mass were electronmicroscopically surveyed at the different stages of spore formation. As a result, it was revealed that the unit membrane of a PSV fused with the plasma membrane, and that the interior membraneous structure was excreted to cover the outside of the plasma membrane (Fig. 3). The filamentous aggregate in the PSV was also extruded into the intercellular spaces. Such excretion of the PSV took place in various parts of the prespore cell, and in due time the whole cell surface was covered by a single layered, electron dense membrane which was originally contained in the PSV. The membrane which covered the outside of the plasma membrane was soon elevated from the plasma membrane during the final stage of spore differentiation, thus completing formation of the spore coat. These observations are also justified by the fact that the outermost membrane of the spore coat had an appearance

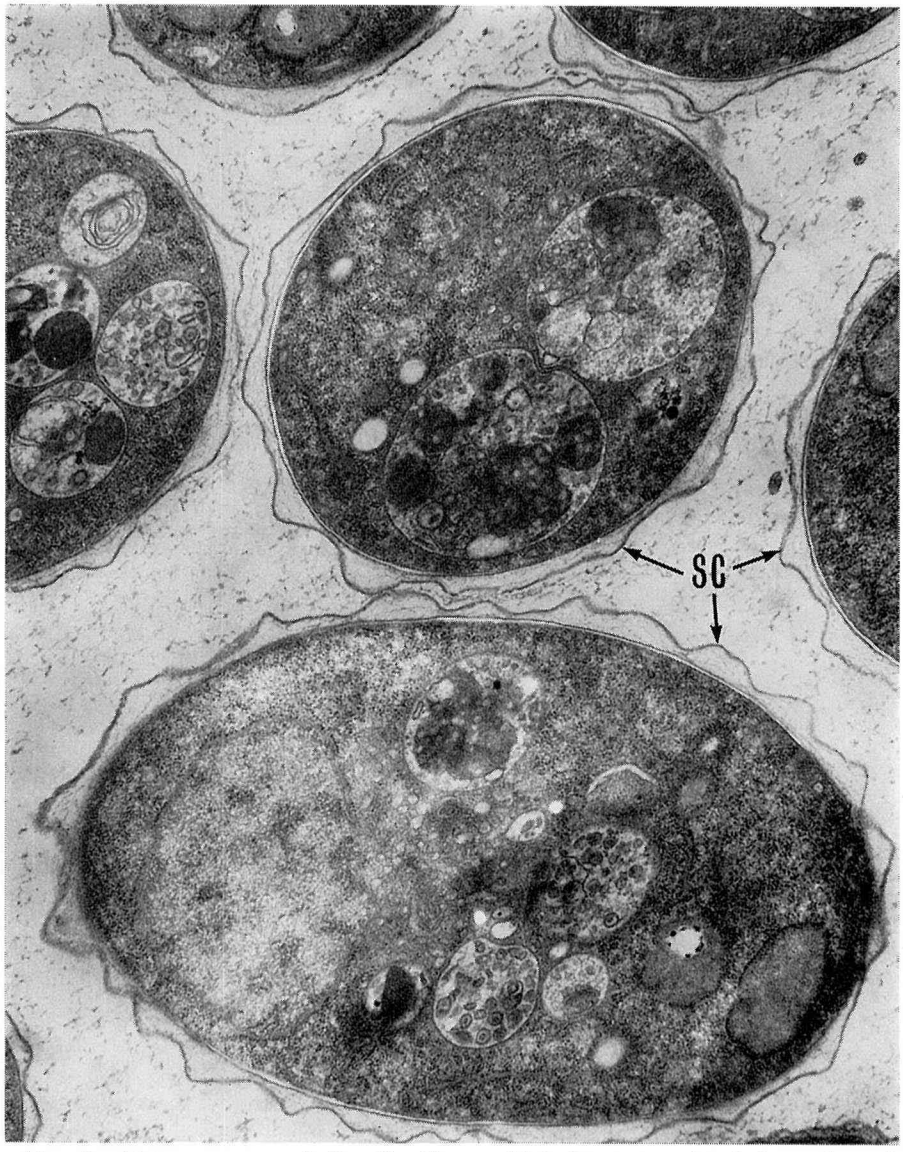

Fig. 2. Mature spores of *D. discoideum* which have completed formation of spore coat (SC). They are devoid of any PSV. X 12,000.

similar to the lining membrane of the PSV. Almost the same observations have been independently obtained by Hohl and Hamamoto (1969).

3. *Formation of the PSV*

In order to investigate how and when the PSV is formed, cells from a late stage of aggregation to an early stage of slug formation were electronmicroscop-

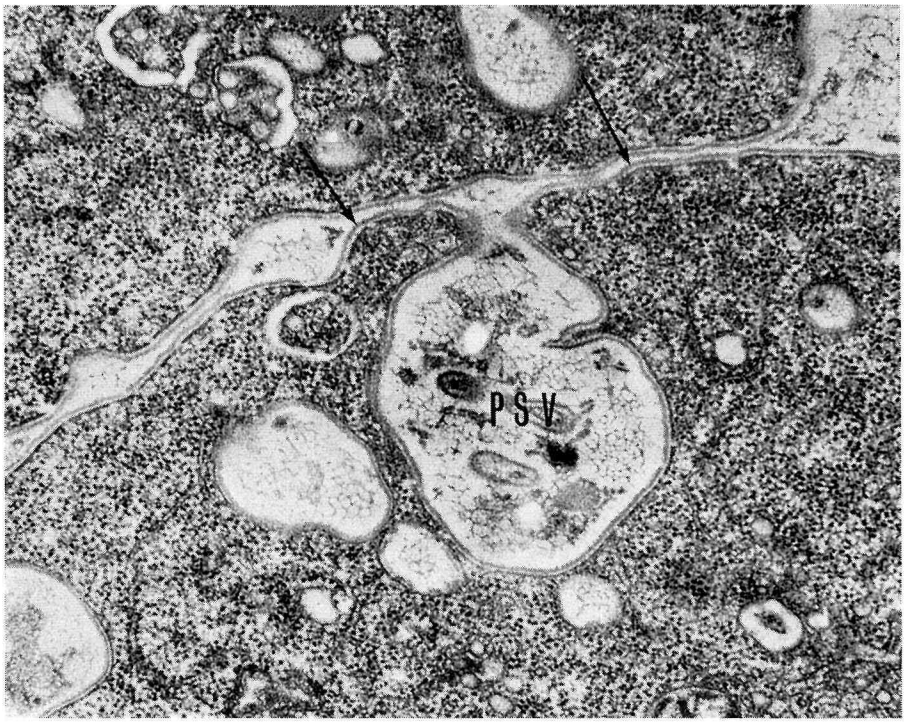

Fig. 3. Excretion of a PSV from a prespore cell at culmination stage. The unit membrane of the vacuole fuses with the plasma membrane, and its lining membrane (arrows) covers a part of the plasma membrane. X 30,000.

ically observed. The various stages of PSV formation were observed, as exemplified in Fig. 4. From these observations, formation of the PSV seems to proceed in the following way. A number of fibers of ca. 50 Å diameter and granules of ca. 200 Å diameter emerges in some vacuoles of 0.5-0.7 μ diameter. The granules are soon arranged in a line along the unit membrane of the vacuole. In due course of development, the granules combines mutually, resulting in formation of continuous membraneous structure in the vacuole.

In some vacuoles, membraneous fragments were actually observed along the inside of the vacuole membrane, indicating transitional stages of formation of continuous membraneous structure (Fig. 5). In all the vacuoles which had completed formation of continuous membraneous structure, only an aggregate of fibers (but no granules) was observed within the membraneous structure.

4. *Chemical nature of spore-specific antigens*

A fluorescein conjugated antiserum produced against spores of *D. mucoroides* stained specifically cytoplasmic granules in the prespore cells of *D. discoideum* (Fig. 6A). The same serum stained only the cell surface of mature spores of

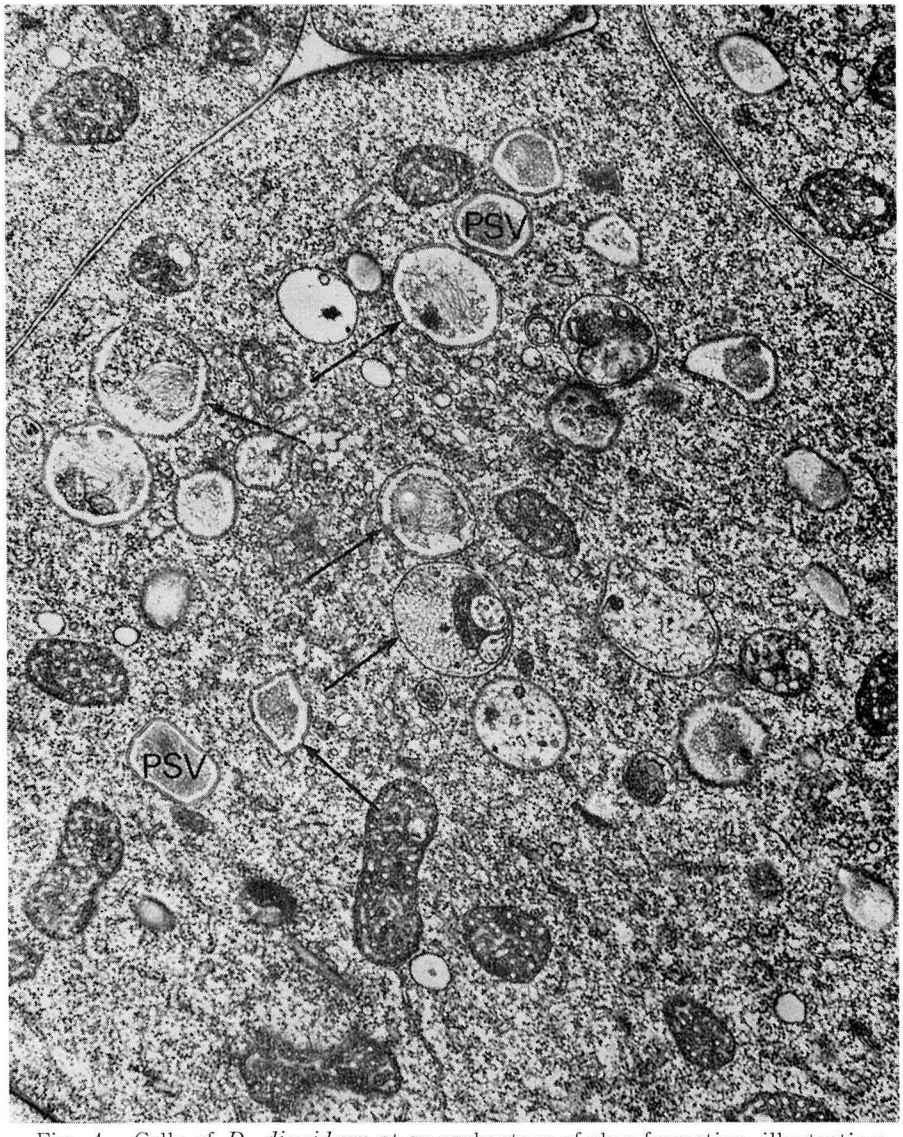

Fig. 4. Cells of *D. discoideum* at an early stage of slug formation, illustrating various stages of PSV formation. Many vacuoles (arrows) are in the process of PSV formation. X 13,300.

the same species (Fig. 7A). These confirmed the results previously obtained by Takeuchi (1963). According to White and Sussman (1963), an acid mucopolysaccharide(s) isolated from fruiting bodies of *D. discoideum* reacts with a homoplastic antispore serum, producing a single precipitin band by the agar

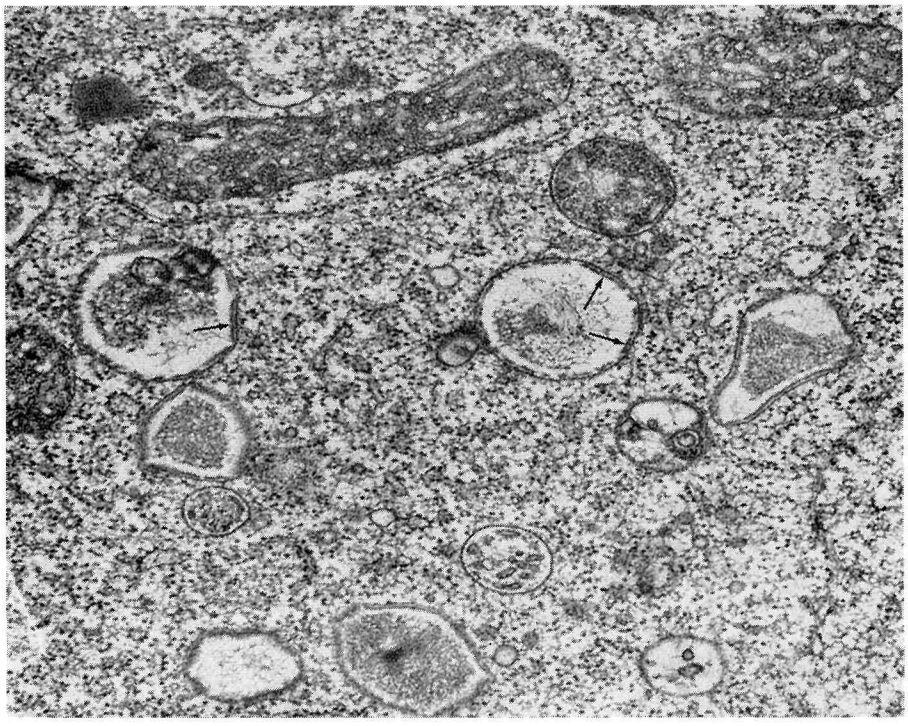

Fig. 5. A highly magnified electronmicrograph of cells of the same stage as Fig. 4. Fragments (arrows) of membraneous structure are observed inside of the unit membrane of the vacuoles. X 26,700.

diffusion technique. Thereupon, a fluorescein conjugated antiserum againt the spores of *D. mucoroides* was absorbed with the acid mucopolysaccharide (s), isolated from fruiting bodies of *D. discoideum*. Prespore cells and mature spores of *D. discoideum* were stained with the absorbed antiserum. No parts of either cells were stained (Fig. 6 B and 7 B). This indicates that a major component of spore-specific antigens is the acid mucopolysaccharide(s).

5. *Galactose incorporation into the cells of the slug.*

According to White and Sussman (1963), a purified preparation of the acid mucopolysaccharide(s) is composed of galactose (54%), galactosamine (25%), and galacturonic acid (9%). In order to examine whether or not synthesis of the acid mucopolysaccharide(s) was limited to the prespore cells of the slug, incorporation of galactose into the slug cells was autoradiographically investigated. Washed amoebae of *D. discoideum* were incubated in standard salt solution containing 50 μCi/ml of ^{3}H-galactose. After formation of slugs, their autoradiographs were prepared, as described in the preceding section. A large number of silver grains were found in the posterior prespore region, while only a few in the anterior prestalk region of a slug (Fig. 8). These facts clearly

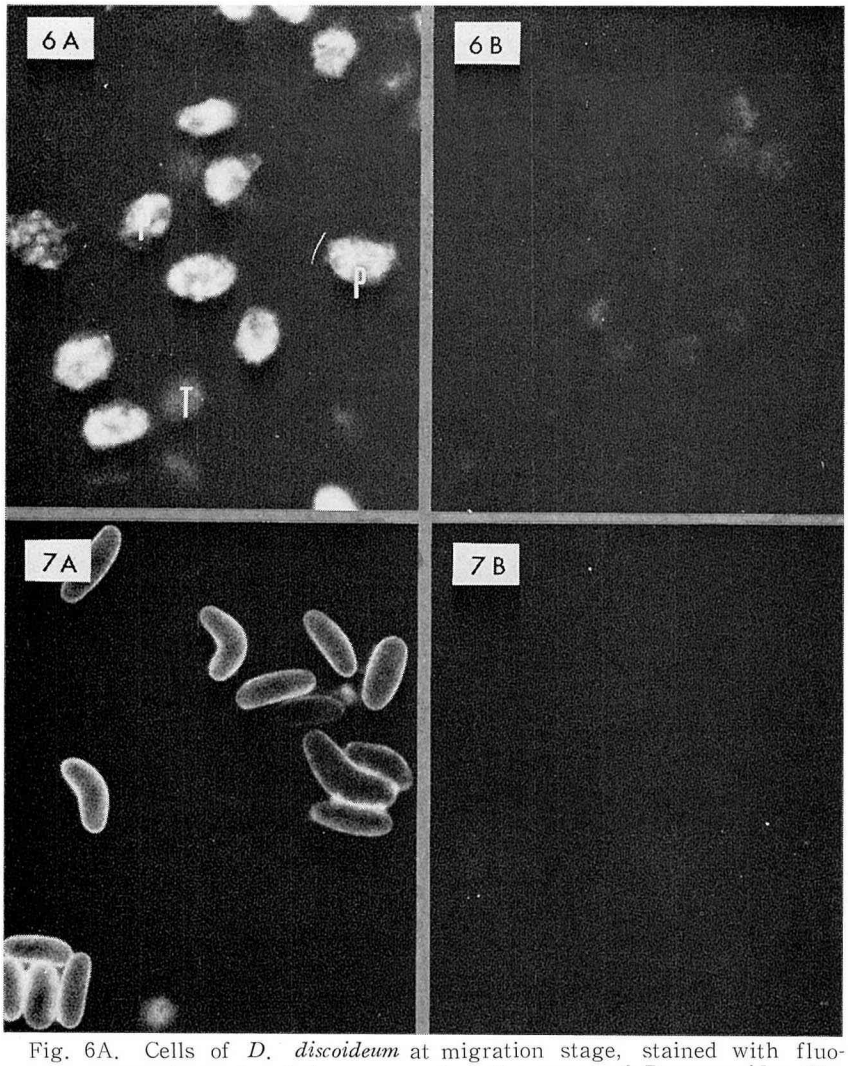

Fig. 6A. Cells of *D. discoideum* at migration stage, stained with fluorescein conjugated serum against the spores of *D. mucoroides*. The staining is limited to cytoplasmic granules of prespore cells (P), but no staining is observed in prestalk cells (T). X 1,320.

Fig. 6B. Slug cells stained with the fluorescein conjugated heteroplastic antispore serum that was absorbed with the acid mucopolysaccharide (s) from the fruiting body. The staining of cytoplasmic granules in prespore cells is almost completely eliminated. X 1,320.

Fig. 7A. Mature spores of *D. discoideum* stained with the fluorescein conjugated heteroplastic antispore serum. The spores are stained only on their surface. X 1,320.

Fig. 7B. Mature spores stained with fluorescein conjugated heteroplastic antispore serum that was absorbed with the acid mucopolysaccharide (s) from the fruiting body. The staining of the spore surface is completely eliminated. X 1,320.

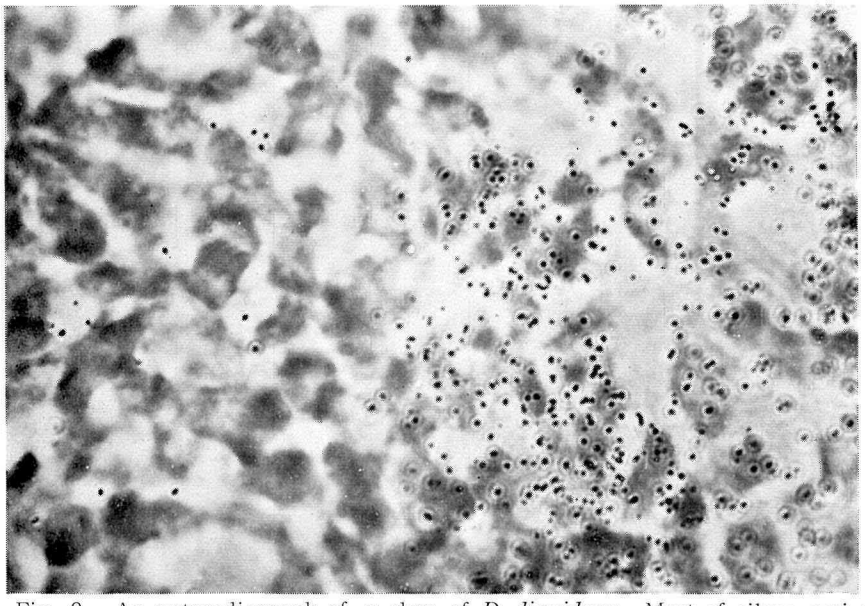

Fig. 8. An autoradiograph of a slug of *D. discoideum*. Most of silver grains are localized in the prespore region, and only a few grains are observed in the prestalk region. X 2,000.

showed that incorporation of galactose into some macromolecules was limited to the prespore cell.

Discussion

It was shown that a number of unique vacuoles named PSV were specifically present in the prespore cells, but not at all in the prestalk cells of the slug. On the other hand, spores which had completed coat formation were devoid of any PSV. During cytodifferentiation from the prespore cell to the spore, it was found that the PSV moved to the periphery of the cytoplasm and fused with the plasma membrane, and that its contents were released and constituted the spore coat. It has been demonstrated that a fluorescein conjugated antiserum produced againt the spores of *D. mucoroides* stains specifically cytoplasmic granules in the prespore cells of the slug of *D. discoideum*, but that in the case of mature spores only the cell surface is stained with the same serum (Takeuchi, 1963). Those cytoplasmic granules in the prespore cell seem to be identical with the PSV, considering from their size and distribution as well as their behavior during spore formation. In fact, fractionation of the prespore cells proved that the PSV was a sole structure in a cell fraction, which gave specific staining with the heteroplastic fluorescein conjugated antispore serum (Ikeda and Takeuchi, unpublished).

It was found in the present study that the heteroplastic antispore serum absorbed with acid mucopolysaccharide(s) of the fruiting body stained neither

the cytoplasmic granules in the prespore cells, nor the surface of spores. From this, it was concluded that a major component of the spore-specific antigens is the acid mucopolysaccharide(s). This conclusion is also supported by the finding that specific staining of the prespore cells with the heteroplastic antispore serum is inhibited in the presence of galactose, galactosamine, and galacturonic acid, which are major sugars constituting the acid mucopolysaccharide(s) (Sakiyama and Takeuchi, unpublished).

Galactose which is a major component of the acid mucopolysaccharide(s) was autoradiographically found to be incorporated specifically into the prespore cells of the slug. Such a specific incorporation of galactose suggests that the acid mucopolysaccharide(s) is synthesized in the prespore cells. In fact, it has been revealed that most of the radioactivities are counted in the fraction of acid mucopolysaccharide(s) specifically precipitated by the heteroplastic antispore serum (Maeda and Takeuchi, unpublished). This fact is in good agreement with the finding that the activity of UDP-galactose polysaccharide transferase which involves in the synthesis of the acid mucopolysaccharide(s) was limited to the prespore cells of the slug (Newell *et al.*, 1969).

Acknowledgments

The author wishes to express his sincere appreciation to Prof. I. Takeuchi for the guidance and encouragement during the course of this work and in preparation of the manuscript. He is also indebted to Miss A. Adachi and Mr. Y. Sakai for the preparation of the antispore serum.

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