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The Properties of Antibody against Smooth Endoplasmic Reticulum of Melanocytes

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KATO, T. and SEIJI, M. *The Properties of Antibody against Smooth Endoplasmic Reticulum of Melanocytes.* Tohoku J. exp. Med., 1980, **130** (4), 311–320 —— The smooth endoplasmic reticulum (smooth-surfaced membrane, SSM) is quite similar in properties to the cytoplasmic membrane which is known to be antigenic. SSM of the melanoma melanocytes can be isolated from the mouse melanoma as a reasonably pure preparation and it contains tyrosinase. Rabbits were immunized by the SSM isolated from Harding-Passey mouse melanomas. Formation of the antibody was detected by the double immuno-diffusion method. Anti-SSM-serum thus formed was found to react with cytoplasmic membrane in the immunofluorescent test, and also showed the specific cytotoxicity to the mouse melanoma cells. On the other hand, the tyrosinase activity of SSM was not inhibited by this antiserum. ——— smooth endoplasmic reticulum; smooth-surfaced membrane; mouse melanoma; cytotoxic test; tyrosinase activity

A halo or depigmentation has been observed to occur in the adjacent areas of both nevi (leucoderma centrifugum Sutton) and malignant melanoma (Kopf et al. 1965; Clark et al. 1975). This phenomenon may be of an immunologic nature, and it is known that the sera of patients with halo nevi and malignant melanomas were cytotoxic against melanoma cells (Copeman et al. 1973; Copeman and Elliott 1976). On the other hand, the histopathological and electron microscopical features specific to vitiligo are a decrease or loss of melanocytes, an increase of Langerhans cells and the appearance of undifferentiated dendritic cells. It was suggested that the undifferentiated dendritic cells are effete melanocytes without function (Mishima et al. 1972). If these changes are brought about through the immunologic mechanisms, it would be necessary that the antibody against cellular components of the melanocyte is formed and melanin formation which takes place in the melanocyte is inhibited.

The smooth endoplasmic reticulum (smooth-surfaced membrane, SSM) is quite similar in properties to the cytoplasmic membrane which is known to be antigenic, and SSM of the melanocyte can be isolated from the mouse melanoma as a reasonably pure preparation and it contains tyrosinase (Seiji et al. 1963).

The present study was designed to examine the antigenicity of the SSM of

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melanocytes in the hope that this experimental study contributes to an understanding of the immunologic aspect of melanocyte in various pigment disorders.

Materials and Methods

Preparation of SSM

According to Seiji et al. (1971), Harding-Passey mouse melanomas, which had been serially transplanted in Swiss mice, were excised and promptly homogenized in nine volumes of 0.25 M sucrose at 0°C with a Porter glass Teflon homogenizer to give a 10% homogenate (w/v). All subsequent procedures were carried out at below 4°C. The homogenate was centrifuged at 13,000 rpm for 10 min, and the resulting supernatant was centrifuged at 40,000 rpm for 60 min. The sediment (small granule fraction) thus obtained was resuspended in 1.32 M sucrose and 2 ml of this fraction was placed at the bottom of the centrifuge tube and 7.5 ml of 1.23 M sucrose and 2 ml of 0.15 M sucrose were layered over it consecutively; then, it was centrifuged at 40,000 rpm for 11.5 hr. The opaque fraction layer over 1.23 M sucrose solution was carefully separated from the packed pellet of rough-surfaced membranes, transferred to a new tube, and diluted with distilled water to reduce the sucrose concentration to 0.25 M. The diluted suspension was centrifuged again at 40,000 rpm for 60 min to precipitate the SSM as a pellet. The SSM sample obtained as a pellet was resuspended in phosphate buffer saline (PBS).

Determination of protein

The protein of SSM and serum was determined by the method of Lowry et al. (1951).

Immunization

Rabbits, 3-month-old and weighing over 2 kg, were used. The rabbits received intramuscular injections of 0.5 ml (1.25 mg protein) of SSM plus an equal volume of Freund's complete adjuvant (DIFCO). Two or three more injections were given at two-weeks-intervals. At two week after the last injection, the rabbits were given 0.5 ml of SSM in PBS and 10 to 14 days later they were bled from the marginal ear veins.

Detection of antibody

The antibody was detected by the double immuno-diffusion method (Ouchterlony 1968) on slide glasses.

Indirect immunofluorescent test

Cover-slips with monolayer culture of cells were washed for 30 min with PBS and then dried at room temperature. After fixed in aceton for 10 min, the antiserum which had been treated by mouse spleen homogenate was placed on the cover-slips and these cover-slips were incubated in a moisture chamber at 37°C for 30 min.

After incubation, the cover-slips were washed with PBS in a stirrer for 10 min and then dried at room temperature. FITC-anti-rabbit-IgG (F/P ratio, 3.1) was diluted to 1:20 with PBS and one drop of this conjugate was placed on the cover-slips. The cells were maintained in a moisture chamber for 30 min at 37°C and then washed again with PBS in a stirrer for 10 min. These cells were dried at room temperature and mounted on with a cover glass. The specimens were observed with a Nikon Fluophoto microscope using UV excitation. Tissue sections of mouse spleen and liver were treated in the same manner.

Cytotoxicity test

Cells. Harding-Passey mouse melanoma; $C_3 4_1$ cell line isolated from B-16 mouse melanoma (Furuse et al. 1969); skin fibroblasts of Swiss mouse; L-cells; cell line HMV (Kasuga 1975); and TM-1 (Nishihira et al. 1977) isolated from human malignant melanomas were used for the test. The cells were cultured as a monolayer in Eagle minimal

essential medium (MEM) supplemented with 10% fetal calf serum. On the experiment, these cells were treated with 0.1% trypsin and cell number were adjusted to 5×10^6/ml in MEM without fetal calf serum.

Absorption of antiserum and complement. One ml of antiserum was added to about 3 g homogenate of Swiss mouse spleen. After 30 min at room temperature the mixture was centrifuged at 15,000 rpm for 20 min. This supernatant was used as an absorbed antiserum. In the same way, guinea pig serum (MILES) treated with about 1 g homogenate of mouse spleen was used as complement. In another way, a series of test tubes which contained 0.1 ml to 0.6 ml of 1:10 antiserum-PBS were heated at 56°C for 30 min and then 100 μg of SSM were added to each test tube. PBS was added further to these mixtures so as to make up a total volume of 1.0 ml, then these test tubes were centrifuged at 3,000 rpm for 15 min. Each of the supernatants was used as another absorbed antiserum.

Cytotoxicity test. As described by Cho and Nishioka (1971), Antiserum absorbed by mouse spleen and non-absorbed antiserum were heated at 56°C for 30 min and were diluted into a 2 times series by MEM without fetal calf serum. 0.05 ml of antiserum was added to 0.05 ml of cell suspensions and 0.05 ml of complement which was diluted to 1:3 in PBS. These mixtures were maintained at 37°C for 45 min. After reaction, these test tubes were stood at 0°C and 0.1 ml of trypan blue was added to them. Cytotoxic index was calculated as the ratio of the number of dead cells per the number of total cells (viable cells+dead cells). Two controls were prepared; one is antibody control which contained only antiserum without complement and the other is complement control which contained only complement.

Morphological method. Cells growing in monolayer cultures in a Labteck chamber were added to equal volumes of antiserum and complement in the same manner as described above and were maintained at 37°C in 5% CO_2 for 3 hr.

Assays of tyrosinase activity

Dopa reaction. On the surface of the double-diffusion gel, filter paper which was soaked in 1 mg/ml of L-dopa and half dried, was pressed and left for 2 hr in a moisture chamber at 37°C.

Assay of tyrosinase activity in vitro. The activity of tyrosinase was measured colorimetrically using 1 mg/ml of L-dopa as a substrate in potassium phosphate buffer, pH 6.8, in final total volume of 3.0 ml (Shimao 1962). Each reaction mixture contained 100 μg of SSM form Harding-Passey mouse melanoma, 1:10 antiserum-PBS or non-immunized rabbit 1:10 serum-PBS and buffer in total volume of 2.4 ml. The amounts of protein of antiserum and non-immunized serum were 40 mg/ml and these sera were heated at 56°C for 30 min before diluted to tenfold. The mixtures were incubated at 37°C for 1 hr and stood at 4°C for 24 hr. Then, these test tubes were centrifuged at 3,000 rpm for 15 min. To each supernatant, 0,6 ml of L-dopa solution was added and the absorbance of the reaction mixtures as measured colorimetrically by a spectrophotometer at 475 nm which is a characteristic absorbance of dopa-chrome. The enzyme activity was expressed as OB/min in a lineary increasing part and the inactivation of tyrosinase by antisera was expressed as the percentage of the enzyme activity of SSM-treated with non-immunized control rabbit serum.

Assay of tyrosinase activity in cultured cells. By the Oikawa-Pomerantz method (Oikawa et al. 1971), tyrosinase activity in cultured cells was determined. $C_3 4_1$ cells were grown in 1.5 ml of culture medium for 3 days. After medium thawed, 1.5 ml of refresh culture medium containing 2μCi/ml of L-tyrosine-3,5-^{3}H and antiserum or non-immunized rabbit serum were incubated at 37°C for 2 hr. To 0.5 ml of thawed medium, 0.1 ml of 1 M trichloroacetic acid TCA and about 50 mg of Norit A were added and mixed well. After standing for 30 min at room temperature, the suspensions were filtered through a Millipore filter and the radioactivity of 0.1 ml of the filtrate was counted under a liquid scintillation spectrometer.

Results

In the double immuno-diffusion (Ouchterlony) test, there were two or three more precipitation lines between SSM treated with 0.1% dioxycholate DOC and antiserum (Fig. 1); one clear line and a few faint lines were observed. Dopa reaction of this clear precipitation line was positive.

From these experimental results, the antibody against the SSM isolated from Harding-Passey mouse melanoma was found to have been formed in the rabbit serum and the results obtained in the double immuno-diffusion method indicate that there are several antibodies against the SSM components. Antiserum prepared was shown to be reactive with the cytoplasm of mouse melanoma cells. As shown

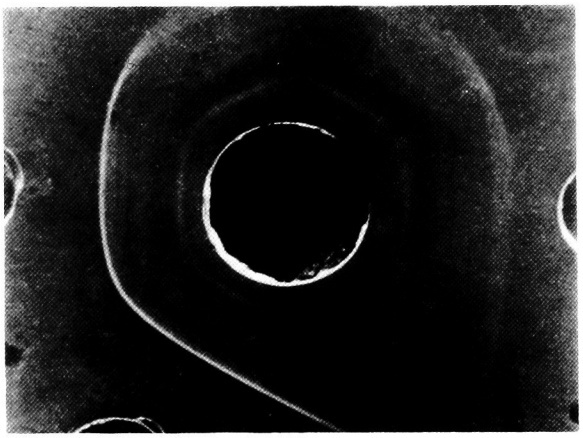

Fig. 1. Results of double immuno-diffusion method. Several precipitin lines were observed between antiserum (1:4) and SSM (from 1:4 to 1:64).

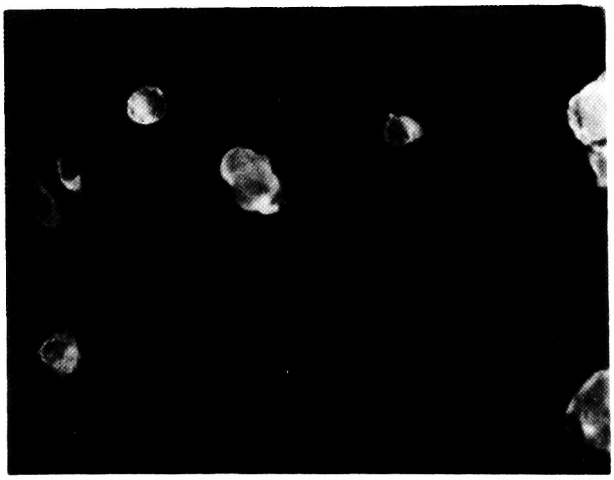

Fig. 2. Immunofluorescence of $C_3 4_1$ cells. Cytoplasms were stained by the treatment with antiserum (1:20) and FITC-anti-rabbit-IgG.

in Fig. 2, the cytoplasm of the C_34_1 cells was stained yellow-green with a 1:20 antiserum-PBS absorbed by mouse spleen homogenates. On the other hand, L-cells, mouse skin fibroblasts, HMV cells or TM-1 cells were not stained with a 1:4 antiserum-PBS.

It was noticed that the non-absorbed antiserum against the SSM isolated from mouse melanoma tissue possessed a cytotoxic effect on the L-cells (Table 1), although it was much weaker than that against the mouse melanoma cells. Antiserum absorbed by mouse spleen, however, did not show any cytotoxicity to the L-cells (Table 2). Antiserum absorbed by 100 μg of SSM also showed the cytotoxicity to mouse melanoma cells in proportion to the concentration of antiserum added (Table 1). Figs. 3 and 4 show the morphological changes of Harding-Passey mouse melanoma cells produced by the treatment with antiserum

TABLE 1. *Cytotoxicity tests*

Antiserum	Dead cells/ dead cells+viable cells (%)		
	C_34_1 cell	H-P cell†	L-cell
1:128*	57	51	37
1: 64*	74	77	47
1: 32*	82	84	66
1: 16*	92	97	69
0.1ml‡		28	
0.2ml‡		39	
0.3ml‡		48	
0.4ml‡		57	
0.5ml‡		65	
0.6ml‡		73	

* Non-absorbed antiserum.
† Harding-Passey mouse melanoma cultured cells.
‡ Supernatants after centrifugation of 1 ml of mixtures containing 100 μg of SSM, 0.1 ml to 0.6 ml of antiserum diluted to 1:10, and PBS.

TABLE 2. *Cytotoxicity test*

Dilution of antiserum	Dead cells/dead cells+viable cells (%)		
	H-P cell	C_34_1 cell	L-cell
1:256*	12.06±3.94	6 77±1.05	
1:128	14.83±2.72	12050±0.40	
1: 64	27.99±1.17	30.43±0.44	
1: 32	43.49±2.01	45.13±2.57	
1: 16	52.26±2.33	52.90±1.52	
1: 8	74.20±1.18	76.30±0.59	
1: 4	83.27±1.24	87.47±1.23	9.63±0.26
Ab control‡	3.84±0.22		2.93±0.69
C control§	8.22±0.35		7.70±0.22

* Absorbed antiserum by spleen.
† Harding-Passey mouse melanoma cells in culture.
‡ Antibody control. § Complement control.

absorbed by mouse spleen. Fig. 3 shows the control and Fig. 4 is the result of treatment with 1:8 antiserum-PBS. No morphological changes were observed among cells grown in MEM containing antiserum without complement, or containing complement without antiserum. On the other hand, when cells were incubated with antiserum and complement, they lost their dendrites, swelled and became round in the shape and also their number decreased significantly in proportion to the concentration of antiserum added. Antiserum was cytotoxic also to the $C_3 4_1$ cells, but not cytotoxic to L-cells, skin fibroblast from mouse, HMV cells or TM-1 cells.

Results of tyrosinase activity of SSM in vitro are shown in Fig. 5. When increasing amounts of antiserum were added, tyrosinase activity in the supernatant decreased linearly with increasing amounts of anti-serum-enzyme complex. In the

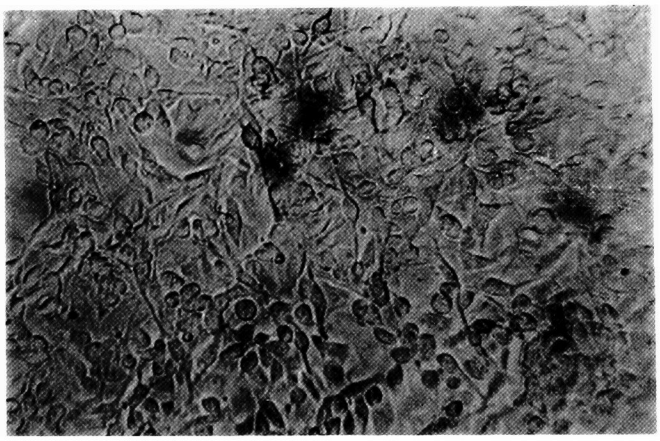

Fig. 3. No morphological changes of Harding-Passey mouse melanoma cells were observed, when treated with antiserum without complement.

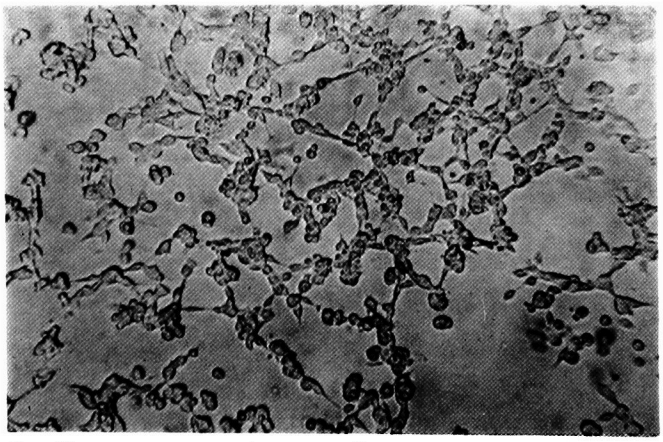

Fig. 4. Harding-Passey mouse melanoma cells were damaged by the treatment with antiserum and complement.

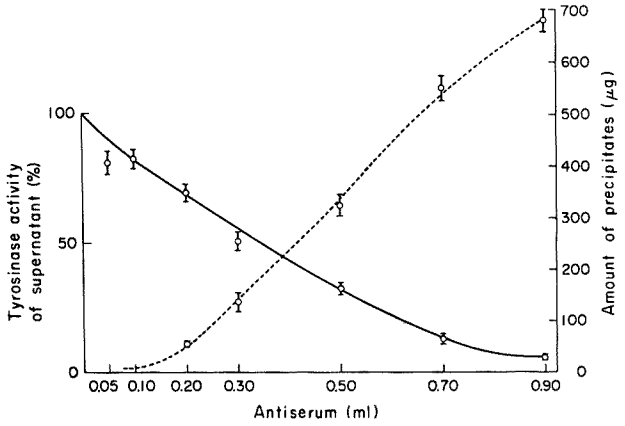

Fig. 5. Tyrosinase activity of supernatant and the amounts of precipitates.

control experiments, non-immunized rabbit serum and buffer did not show the decrease of tyrosinase activity. The tyrosinase activity of the SSM did not change after mixed and incubated with 2.0 ml of antiserum (2 mg protein), enough to precipitate the total enzyme. And precipitates obtained after centrifugation showed to possess the tyrosinase activity when treated with 0.1% DOC.

The Oikawa-Pomerantz method allows us to determine the melanin forming activity of the cultured melanocytes. Tyrosinase activity of cells grown in MEM containing non-immunized rabbit serum was 3960 cpm and that growth in MEM containing 1/16 antiserum solution was 4434 cpm. There was no significant difference between antiserum group and nonimmunized rabbit serum group. The calculation of melanin forming activity is based on per same cell number (10^5 cells) of melanocytes in cell culture.

Discussion

An attempt was made to prepare the antibody against the smooth-surfaced membrane in order to study on the immunological aspects of melanocytes. Concerning the antigenicity of melanocytes, a question arises as for which cell constituents of the melanocytes could be antigenic to the host. Cytoplasmic membrane and melanosomes would more likely be antigenic elements among others such as mitochondria, ribosomes, endoplasmic reticulum, nuclei and so on. The antigenicity of melanosomes has been examined with melanosomes isolated from black hair in standard immunization technique. The melanosomes thus prepared did not contain any outer membranes of melanosomes which might be important for antigenicity. Such an attempt to demonstrate rabbit antibodies specific to human hair melanosomes was unsuccessful (Ogawa, personal communication).

Soluble tyrosinase was isolated from the melanosome fraction of B-16 mouse melanoma and rabbit antiserum against B-16 mouse tyrosinase was prepared by the standard immunization thechnique. The anti-tyrosinase serum thus prepared

was specific to tyrosinase and it was also found that the tyrosinase-antiserum-precipitate showed the tyrosinase activity. Antiserum against tyrosinase did not inhibit the melanin formation (Ohtaki and Miyazaki 1972).

Cytoplasmic membranes of melanoma cells have not been isolated yet as a pure fraction because of the chemical and technical difficulties. On the other hand, the smooth-surfaced membrane of the endoplasmic reticulum has been prepared as a reasonably pure fraction from mouse melanoma (Seiji and Iwashita 1965) and its properties are assumed to be quite similar to those of the cytoplasmic membrane. This fraction contains tyrosinase activity.

Non-absorbed antiserum was cytotoxic not only to mouse melanoma cells but also to mouse fibroblasts. This might be interpreted as follows: Harding-Passey mouse melanoma contains some fibroblasts or histiocytes, therefore, the SSM prepared could be derived both from melanoma cells and from fibroblasts. Thus, non-absorbed antiserum might show the cytotoxicity to mouse fibroblasts, although its effect was much weaker than that against mouse melanoma cells. And antiserum absorbed by mouse spleen homogenates did not show the cytotoxicity to mouse fibroblasts. This serum was not cytotoxic to human melanoma cells. Therefore, the antiserum absorbed by mouse spleen homogenate seems to be specifically cytotoxic to mouse melanoma cells, although its cytotoxic effect as weaker than that of non-absorbed antiserum. This specificity was confirmed by the results of cytotoxicity test using the antiserum which was pre-treated with 100 μg of SSM.

From immunofluorescence studies, the specificity of this antiserum cannot be discussed because the F/P ratio of FITC-anti-rabbit-IgG used was high. But it was shown that this antiserum possessed an antigenicity against cytoplasm of the melanoma cell.

Tyrosinase in SSM seems to exist as an element of the membrane structures (Seiji et al. 1963). Therefore, antibody against tyrosinase might be formed in antibody against the SSM of melanoma cells. The SSM contains acid phosphatase, APT-ase and glucose-6-phosphatase besides tyrosinase, and these enzymes in SSM seem to exist also as elements of the membrane structure. In Ouchterlony's method, two or three more precipitation lines were observed and this is interpreted by the presence of such components in SSM. The precipitation line was positive in the dopa-reaction and it was suggested that the antiserum might not inhibit the melanin formation. The results of the determination of tyrosinase activity in vitro confirmed this possibility. Antibody against SSM could bind with SSM but did not inhibit the tyrosinase activity. With these respects, the antibody produced was similar to that against soluble tyrosinase prepared by Ohtaki and Miyazaki (1972) and that against mushroom tyrosinase by Adams (1942). From their and our results, it is suggested that the active site of tyrosinase and the antigenic site of the SSM might be different and located apart each other.

On the aspect of humoral immunity, therefore, it is not likely that antiserum against cellular components, such as tyrosinase, might prevent the melanin formation. And if such a humoral antibody as this is related to the depigementation of

the skin in the autoimmune mechanism, only cytopathic effect of the cytoplasmic antibody would take place.

Acknowledgment

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