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Antibody formation for malignant tumor. Ⅲ. Antigenicity of peptide of ribosomal digest in Ehrlich ascites tumor*

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Abstract

Antigenicity of the peptide of ribosomal digest in Ehrlich ascites tumor was studied. The peptide was purified by DEAE Sephadex A-50 column chromatography. The peptide was electrophoretically basic, single, and 1.32 S_{20w} sedimentation coefficient with poor content of tyrosine and phenylalanine. The maximum absorbancy was at 267 m μ . Mice and rabbits were immunized with the mixture of the purified peptide with Freund's complete adjuvant. The inhibitory effect of immune γ -globulin on the tumor growth was demonstrated in vitro cultured JTC-11 cells. A single precipitin line was observed between rabbit antiserum and tumor cell extract of Ehrlich ascites cells in Ouchterlony double diffusion chamber and immunoelectrophoresis. The sedimentation coefficient of the effective fraction in immune-serum was 17 S_{20w}. The precipitin line was observed at β 2- γ region in immunoelectrophoresis.

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ANTIBODY FORMATION FOR MALIGNANT TUMOR
III. ANTIGENICITY OF PEPTIDE OF RIBOSOMAL
DIGEST IN EHRlich ASCITES TUMOR

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Antitumor activity in regional lymph node protein of mice transplanted with Ehrlich ascites tumor was reported (1). The antigenicity of tumor low density lipoprotein was demonstrated in the previous experiment, which was clarified that the phospholipids of low density lipoprotein have an important role for the antibody formation (2).

In the present experiment, the specific peptide of the ribosome of Ehrlich ascites tumor was studied, and the antitumor activity of the mouse serum immunized with this peptide was demonstrated JTC-11 cells cultured *in vitro* and immuno-diffusion technique.

MATERIALS AND METHODS

Materials: Albino dd mice were inoculated subcutaneously with JTC-11 cells (3) as described previously (1). The tumors on the tenth day after transplantation were extirpated, minced in Waring blender, and then homogenized in a glass-Teflon homogenizer with 10 volumes of 0.05 M Tris-HCl buffer containing 0.025 M KCl and 0.005 M MgCl₂, pH 7.6. The homogenate was centrifuged twice at 12,000 × g for 40 min. in a Hitachi Ultracentrifuge Model 40P at 2°C, and the supernatant was centrifuged at 50,000 × g for 50 min. The precipitate was resuspended with 20 ml of Tris-HCl buffer, and 8 % sodium desoxycholate was added to give the final concentration of 0.8 %. The suspension was allowed to stand for 2 hrs at room temperature, and centrifuged at 144,000 × g for 1 hr. The precipitate, yellowish jelly, was crude ribosome. About 100 mg of precipitate of crude ribosome was dialyzed in 0.005 M MgCl₂, suspended in 10 ml of Tris-HCl buffer, and incubated with 15 mg of RNase and 5 mg of DNase. Thus, ribosomal digest was obtained.

Preparation of Peptide: The ribosomal digest solution was fractionated on DEAE Sephadex A-50 column (3 × 30 cm) with 0.05 M Tris-HCl buffer

containing 0.025 M KCl and 0.005 M MgCl₂, pH 7.6, and the elution patterns were recorded at 280 m μ by the autorecording Toyo Uvicon-540 optical unit attached to Toyo Fraction Collector SF-200A as illustrated in Fig. 2. The absorbancy of the fractionated peak was determined by (Model

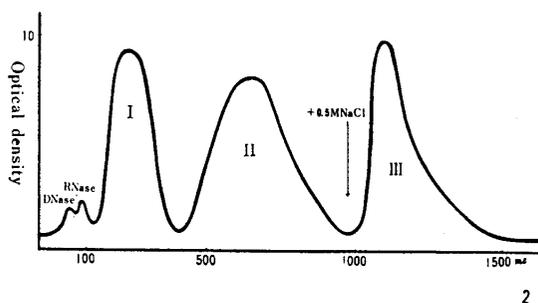


Fig. 2 Chromatography of ribosomal digest on DEAE Sephadex A-50. A 20-ml sample was applied on a 3 \times 30 cm column. Elution was done with 0.05M Tris-HCl buffer containing 0.025 M KCl and MgCl₂, pH 7.6, and the peak I was peptide, and the peak III was eluted by adding 0.5 M NaCl in the same buffer.

EPS-3, Hitachi UV-VIS-NIR) recording spectrophotometer (Model EPS-3, Hitachi UV-VIS-NIR). The maximum absorbancy of the peptide from ribosomal digest was 267 m μ as seen in Fig. 3. Peak I was the peptide of ribosomal digest, and Peaks II and III were RNA, and the peak was subjected to disc electrophoresis (4). The peptide was desalted by Sepha-

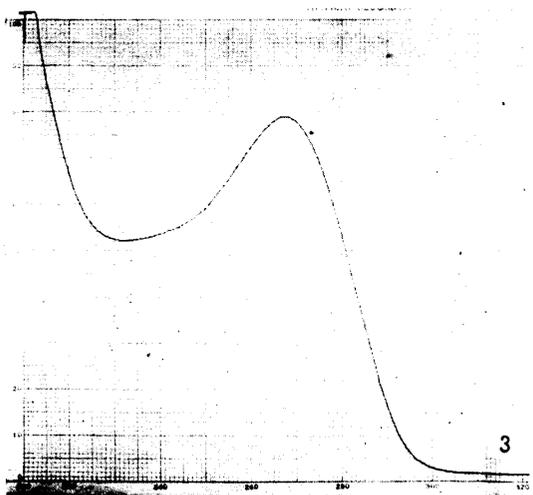


Fig. 3 Absorbancy of the peptide of tumor ribosomal digest. Maximum absorbance of the peptide (Peak I in Fig. 2) was observed at 267 m μ .

dex G-15 column, and was used for amino acid analysis (5). Protein concentration was determined by the colorimetric method of LOWRY *et al.* (6), and the nitrogen content was determined by the micro-Kjeldahl method.

Preparation of Antisera: One mg of the peptide was dissolved in 0.5 ml of saline. The peptide solution was mixed with one-half volume of Freund's complete adjuvant after filtrating through a sterilized millipore filter. The mixture was intraperitoneally injected to dd mouse, and one-half volume of the mixture was subcutaneously added weekly for a month. The mixture was injected to rabbit three times weekly. Four injections of 5 mg/ml peptide were done into subcutis of foot and back and in gluteal muscle. Test bleedings were carried out on the 5th day after the last injection, and when the sera were satisfactory the animals were injected intravenously with 5 mg of peptide as a booster. One week later, the rabbits were sacrificed, and the sera were kept at -20°C after addition of merthiolate to make a final concentration 1/5,000.

Cell Culture Test: The antitumor activity of immune serum of mice was examined by cell culture technique, and that of the supernatant of lymph nodes and spleen homogenate was also tested as described previously (1).

Precipitin Reaction of Antisera and Immuno-electrophoresis: Precipitin reaction of anti-serum was performed by the OUCHTERLONY'S technique (7), and immuno-electrophoresis was carried out by the micro-method (8).

RESULTS

Chemical Characters: Ultracentrifugal analyses of purified ribosome and the peptide from ribosomal digest of Ehrlich ascites tumor are shown in Fig. 1. The sedimentation constant was $1.32\text{S}_{20\text{w}}$, and the molecular weight was presumed about 6,000. Disc electrophoresis, OUCHTERLONY'S double diffusion and immuno-electrophoresis of the peptide are shown in Fig. 4. The electrophoretic mobility of the peptide was more basic than that of serum IgG. In these photographs, the peptide from ribosomal digest was demonstrated to be single and probably specific for Ehrlich ascites tumor cells. Amino acid components of the peptide are shown in Table 1. The contents of tyrosine

TABLE 1 AMINO ACID COMPOSITION OF THE PEPTIDE IN TUMOR RIBOSOMAL DIGEST

threonine	4.4 %
serine	8.4
glutamic acid	6.7
glycine	30.7
alanine	4.0
methionine	3.6
isoleucine	2.2
leucine	3.1
tyrosine	1.8
phenylalanine	1.8
lysine	27.6
histidine	5.8

A 5-mg of the lyophilized peptide was treated according to MOORE *et al.* (5), and was analyzed on YANAGIMOTO auto-recording analyzer, Model LC-5.

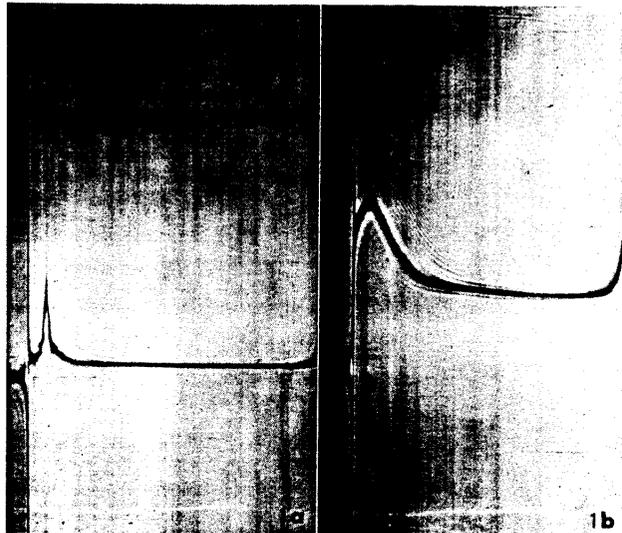
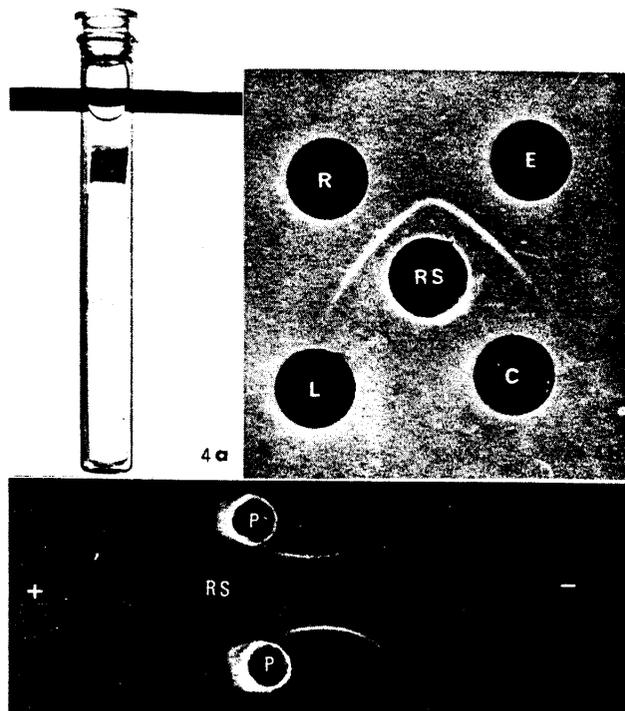


Fig. 1 Ultracentrifugal analyses

- a) Purified tumor ribosome. The peak was observed at 3 min. of 31,820 rpm, and its sedimentation coefficient was 75 S_{20w} .
- b) Peptide of tumor ribosomal digest. The peak was observed at 15 min. of 60,000 rpm, and its sedimentation coefficient was 1.32 S_{20w} .



and phenylalanine residues were small quantities.

Cell Culture Test: Growth inhibitory effects of globulin fraction from immune serum and the supernatant of lymph nodes and spleen homogenate of immunized mice are shown in Fig. 5. The effects on the antitumor

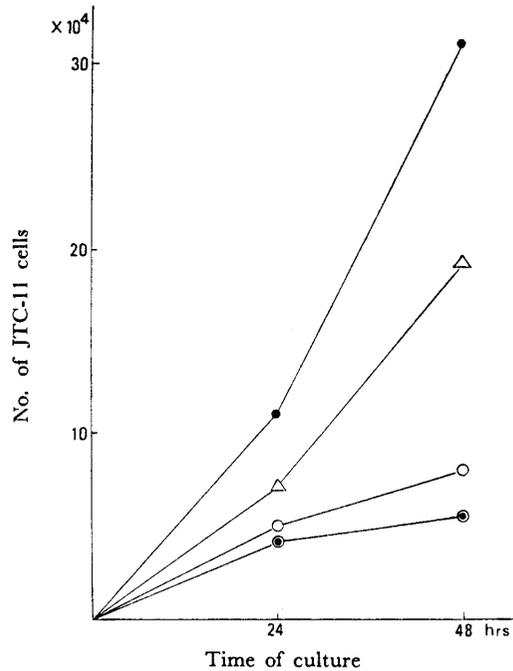


Fig. 5 Antitumor activity of immunoglobulins.

Normal mouse serum globulin, immune globulin, and the supernatant of immunized lymph nodes and spleen homogenate were added each in protein concentration of 1 mg/ml for each cell culture tube.

●=control, △=normal mouse serum globulin, ○=supernatant of lymph nodes and spleen homogenate immunized with the peptide of tumor ribosomal digest, ⊙=serum globulin immunized with the peptide of tumor ribosomal digest.

Fig. 4. Electrophoresis of the peptide

a) Disc electrophoresis

The peptide was analyzed on a disc gel column, and a single band was observed in the middle of the column.

b) Precipitin reaction of anti-serum

Single precipitin line was observed among ribosome, Ehrlich ascites tumor homogenate and rabbit anti-serum.

R = ribosome of Ehrlich ascites tumor, E = Ehrlich ascites tumor homogenate, L = normal mouse liver homogenate, G = normal mouse serum, RS = rabbit anti-serum to ribosome peptide.

c) Immuno-electrophoresis

A single line was observed from β_2 to γ position.

Rs = rabbit anti-serum, P = peptide of tumor ribosomal digest.

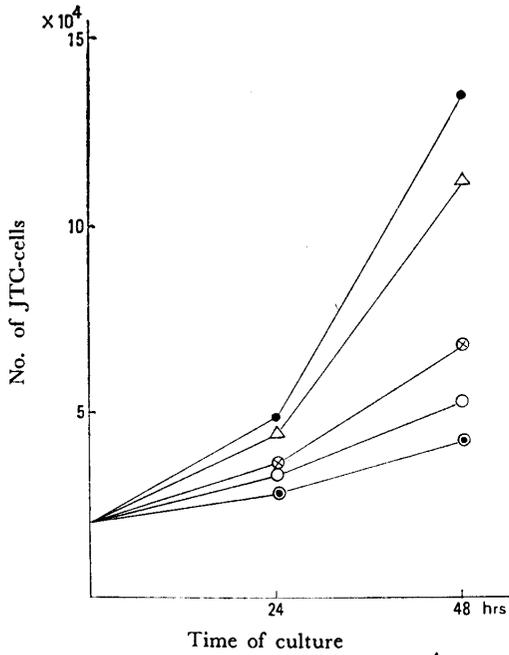
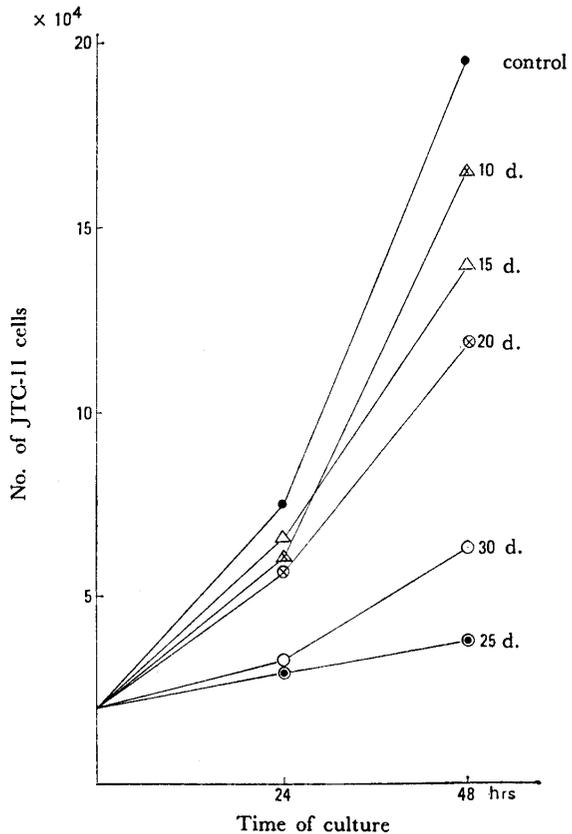


Fig. 6 Antigenicity and peptide concentration immune globulin at 25th day after immunization, obtained by immunizing with various concentrations of the peptide.

⊙=1 mg, ○=0.5 mg, ⊗=0.25 mg, △=normal mouse serumglobulin, ●=control.

Fig. 7 Antitumor activity and days after immunization immune-serum was obtained on various days after immunization, and each 1 mg/ml protein concentration was added for cell culture tube. d=days after immunization



activity of the peptide concentration used as antigen and the days after immunization are shown in Figs. 6 and 7. Sera at the 25th day immunized by 1 mg peptide were found to have the highest antitumor activity. The growth inhibitory effect was mostly exist in γ -globulin (Fig. 8).

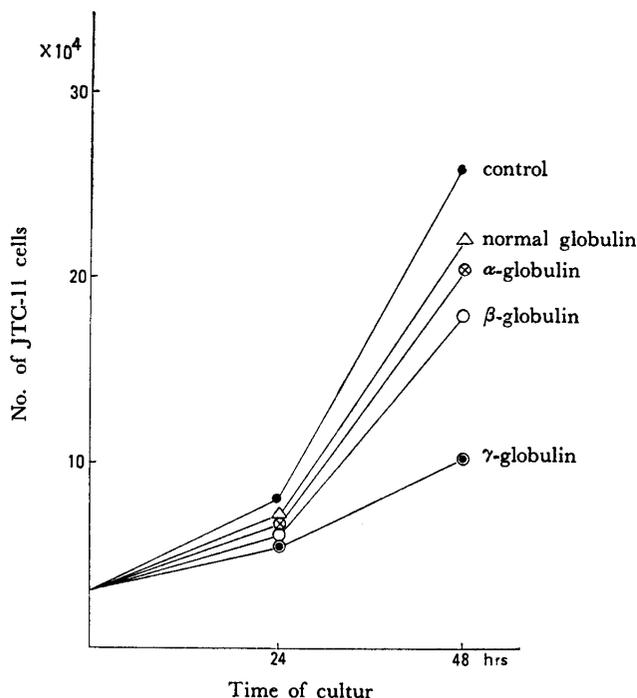
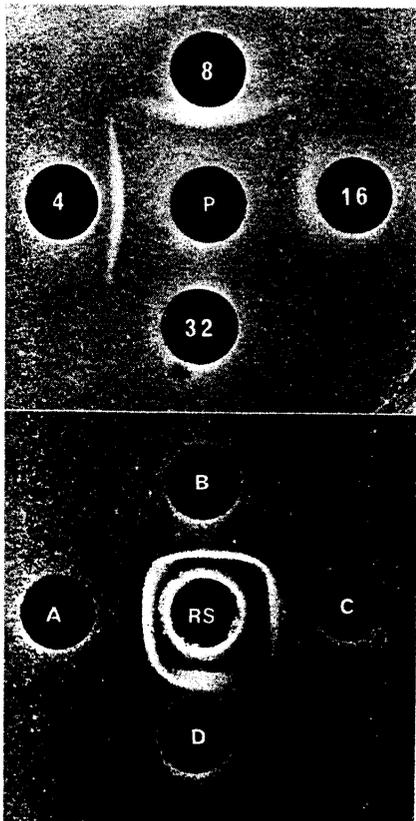


Fig. 8 Antitumor activity of immune. γ -globulin α -, β - or γ -globulin of immune-serum was added for cell culture tube. Immune-serum was fractionated on DEAE Sephadex A-50 column, and 1 mg/ml protein concentration of each α -, β - and γ -globulin fraction was added for cell culture tube.

Titer of Antiserum and Antigenicity of Peptide: The titer of antiserum and antigenicity of the peptide were determined by ouchterlony's technique, as seen in Fig. 9. Precipitin line was observed in 24-fold dilution. Antigenicity was tested by multiple dilution of the peptide solution in ouchterlony double diffusion chamber, and a sharp precipitin line was observed at 0.5 mg/ml protein concentration. Quantitative precipitin reaction was carried out according to HEIDELBERGER and KENDALL (9), and the results are shown in Fig. 10. The equivalent zone of antibody was inclined to yield antibody excess.

Ultracentrifugal Analysis of γ -Globulin: γ -Globulin of rabbit anti-serum was chromatographed on Sephadex G-200 column (1.5×120 cm), and



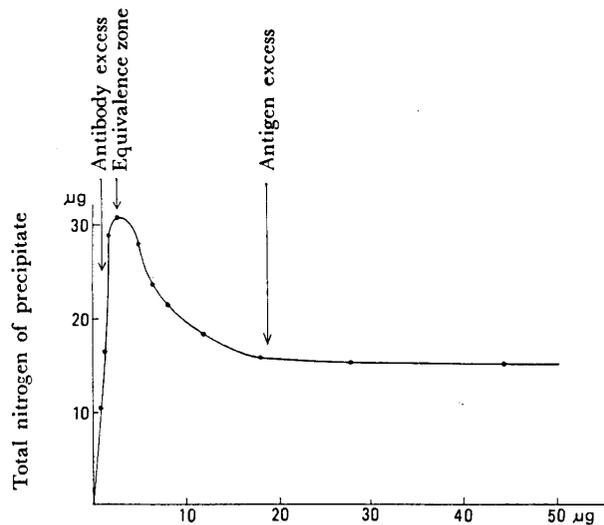
the first elution peak was analyzed in a Hitachi Analytical Ultracentrifuge Model UCA-I as shown in Fig. 11. The sedimentation coefficient was $17.05 S_{20w}$.

DISCUSSIONS

MITCHINSON (10), ROSENAU (11) and KLEIN (12) revealed the cellular antibody for cancer, and in the previous report (1), the possibility of

Fig. 9 Titer of anti-serum and antigenicity of peptide

a) Titer of anti-serum
 P = peptide of tumor ribosomal digest. 4, 8, 16, and 32 = Numbers of fold dilution of rabbit anti-serum
 b) Titer of antigen
 RS = rabbit anti-serum, A = 1 mg/ml of the peptide from tumor ribosomal digest, B = 0.5 mg/ml of the peptide, C = 0.25 mg/ml of the peptide, D = 0.125 mg/ml of the peptide.



Nitrogen of the peptide added to 0.5 ml of rabbit anti-serum

Fig. 10 Quantitative precipitin reaction

Quantitative precipitin reaction was determined by total nitrogen of the precipitate.

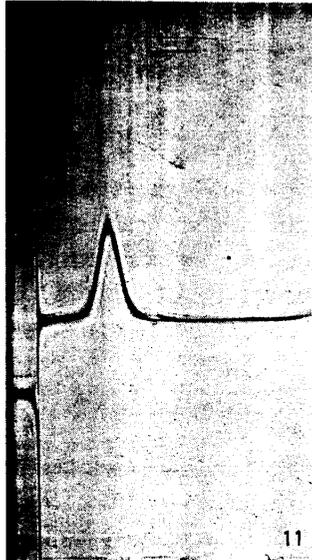


Fig. 11 Ultracentrifugal analysis of the effective fraction of rabbit anti-serum. A peak was observed at 16 min. of 60,000 rpm, and its sedimentation coefficient was 17.05 S_{20w} .

antibody formation for malignant tumor was demonstrated. KORNGOLD (13), PINKOWSKI (14) and ZILBER (15) reported the existence of cancer specific antigen by precipitin reaction. HIRAI also reported the cancer specific antigen of rat ascites Hepatomas (16).

In the present experiment, the antigenicity of the peptide obtained from the ribosome of Ehrlich ascites tumor was proved by *in vitro* cultured JTC-11 cells, and the specificity by precipitin reaction. The peptide from tumor ribosome was electrophoretically single with the absorbancy of 267 $m\mu$. Immuno-electrophoresis and precipitin reaction showed to be specific for Ehrlich ascites cells, but the contamination of nucleic acid cannot be excluded. The content of tyrosine and phenylalanine was small quantities in the amino acid components of the peptide. It is the well-known evidence that tyrosine has an important role for antibody formation (17). The fact indicates the difficulty of antibody formation by the peptide from ribosomal digest of Ehrlich ascites tumor, which was observed in weak sensitivity of the precipitin reaction (Figs. 9, 10).

The ribosome is a manufacture unit of protein synthesis of the cell (18, 19). If cancer cell has a specific protein or antigen, the peptide of the ribosome may have specificity for the cancer cell. In the previous experiment, the antigenicity of low density lipoprotein in JTC-11 cells was

demonstrated. Low density lipoprotein is a structure factor of membrane in the cell (20, 21). Therefore, it is considered in the immunological stand point of view that the cancer cells have two kinds of cell-specificities, one is in the membrane structure and the other in the peptide structure of ribosome. These two kinds of specificities might be occupying a small part of the cancer cell. Therefore, the antibody formation for malignant tumor was difficult, and spontaneous healing of cancer was rarely observed.

In the present experiment, the possibility of antibody formation for malignant tumor was demonstrated, and the existence of Ehrlich ascites cell-specific antigen was also clarified.

CONCLUSIONS

Antigenicity of the peptide of ribosomal digest in Ehrlich ascites tumor was studied. The peptide was purified by DEAE Sephadex A-50 column chromatography. The peptide was electrophoretically basic, single, and $1.32 S_{20w}$ sedimentation coefficient with poor content of tyrosine and phenylalanine. The maximum absorbancy was at $267 m\mu$. Mice and rabbits were immunized with the mixture of the purified peptide with Freund's complete adjuvant. The inhibitory effect of immune γ -globulin on the tumor growth was demonstrated *in vitro* cultured JTC-11 cells. A single precipitin line was observed between rabbit antiserum and tumor cell extract of Ehrlich ascites cells in Ouchterlony double diffusion chamber and immunoelectrophoresis. The sedimentation coefficient of the effective fraction in immune-serum was $17 S_{20w}$. The precipitin line was observed at $\beta_2\text{-}\gamma$ region in immunoelectrophoresis.

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