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Abstract

A unique low density lipoprotein was obtained from the tumor transplanted with a cultured cell line of Ehrlich ascites tumor, JTC-ll cell. The tumor low density lipoprotein electrophoretically migrated as a single band, and the mobility was different from that of other organs. The chemical composition of lipid, cholesterol and phospholipids in tumor low density lipoprotein were characteristic. The flotation rate was Sf 5.9, and thus the molecular weight was estimated to be about 130 x 104. The inhibitory effect on tumor growth of the immune serum was most elevated at 25th day after the intraperitoneal administration of tumor low density lipoprotein. The main fraction effective for inhibition of tumor growth existed in γ-globulin.

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ANTIBODY FORMATION FOR MALIGNANT TUMOR II. ANTIGENICITY OF EHRLICH ASCITES TUMOR LIPOPROTEIN

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Tumor specific antigenicity has been clarified from the aspect of genetical compatible host. It has been discovered by FOLEY (1) and has been confirmed by PREHN (2), and KLEIN (3) that methylcholanthrene-induced sarcomas in mice are strongly antigenic. They reported that the antigenicity was tumor specific and that it did not depend on genetically determined isoantigenic differences between donor and recipient. In the previous report (4), the antitumor activity of regional lymph node protein of mice transplanted with a cultured cell line of Ehrlich ascites tumor, JTC-11 cell, was demonstrated.

In the present experiment, the low density lipoprotein in tumor of dd mice transplanted with JTC-11 cells was prepared by dextran sulfate precipitation method. Tumor low density lipoprotein showed a single band in electrophoresis, and the chemical characters were different from those of other organs. The growth inhibitory effect was observed *in vitro* cultured JTC-11 cells by the serum immunized with the tumor low density lipoprotein.

EXPERIMENTALS

Materials: Albino dd mice weighing about 20 g were inoculated subcutaneously with a cultured cell line of Ehrlich ascites tumor, JTC-11 cell, as described previously (4). The tumors grown to the size of about a little finger tip on 10th day after transplantation were collected in pool, about 100 g in wet weight, minced in a Waring blender, and then homogenized in a glass-Teflon homogenizer in 10 volumes of 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.6. Tumor homogenate was centrifuged at 140, 000 $\times$ g for 2 hrs in a Hitachi Preparative Ultracentrifuge, Model 40 P. Low density lipoprotein was prepared by dextran sulfate precipitation method from the supernatant solution (5), and high density lipoprotein

was obtained from the precipitate according to the method of MANSON *et al.* (6). Dextran sulfate (molecular weight; about 2×10^5), protamine sulfate and DEAE-Sephadex A-50 were purchased from Pharmacia Uppsala, Sweden. Other reagents used were all of reagent grade purchased from Wako Pure Chemicals Co., Daiichi Pure Chemicals Co. and Merk Co.

Chemical Analysis: The concentration of lipoprotein was determined by the colorimetric method of LOWRY *et al.* (7), combined at 280 mμ. The concentration of low density lipoprotein was corrected by micro-Kjeldahl method using the insoluble peptide residue of delipidated lipoprotein. The factors for correction were 0.76 for serum low density lipo protein, 0.80 for liver, and 0.82 for tumor.

Delipidation: All procedures were carried out at room temperature. One volume of low density lipoprotein was added slowly to 15 volumes of methylal-methanol (4 : 1, v/v), and the solution was allowed to stand for at least 2 hrs. The insoluble part was separated by centrifugation for 15 mins, and it was resuspended twice in 5 volumes of acetone, and finally in 10 volumes of acetone-water (2 : 1, v/v).

Lipid Analysis: Total lipid content was determined by the method of BRAGDON (8). Cholesterol was determined by the procedure SCHOENHEIMER-SPERRY (9, 10), and lipid phosphorus was by FISKE-SUBBAROW (11). The factor 25 was used to convert lipid phosphorus to phospholipid. Neutral lipid was estimated substracting cholesterol and phospholipid from total lipid content. The lipids were separated on thin layer chromatography (12, 13).

Ultracentrifugation: Ultracentrifugal analysis of low density lipoprotein was carried out in about 1.063 density NaCl solution (14). Svedberg identity was estimated from the flotation rate of tumor low density lipoprotein, and the molecular weight was calculated according to LINDGREN *et al.* (15).

Electrophoresis: Disc electrophoresis on polyacrylamide gel was performed at pH 2.3 with a current of 2.5 mA per gel column for 90 mins (16, 17). One of two polyacrylamide gel columns was prestained with Sudan black B, and the other one was stained with amido black after migration.

Immune-serum: Albino dd mice were injected intraperitoneally with 1 mg protein concentration of low and high density lipoprotein, and 0.5 mg of lipoprotein was supplemented subcutaneously every 1 week for month. Immune-serum was obtained on 5, 10, 15, 20, 25, 30, and 40th day after immunization, and kept at −20°C. Immune-serum was separated by

DEAE-Sephadex A-50 column chromatography as described previously (4). The separated fractions were dialyzed in distilled water, and stored at −20°C after lyophilization.

Cell Culture Test: The 2×10^5 or 3×10^5 JTC-11 cells were cultured as described previously (4). The antitumor activity of immune-serum and its globulin fraction were determined *in vitro* cultured JTC-11 cells. The supernatant of lymph node and spleen homogenate tested the antitumor activity on 10th day after the intraperitoneal administration of tumor low density lipoprotein.

RESULTS

Lipid to protein, phospholipid to cholesterol and phospholipid to protein ratio of low density lipoprotein were shown in Table 1. The ratio of

TABLE 1. CHEMICAL CHARACTERS OF LOW DENSITY LIPOPROTEIN

LDL		Total Lipid Protein	Phospholipid Cholesterol	Phospholipid Protein
Normal	Serum	7.4	1.30	1.20
	Liver	4.4	1.22	0.64
	Spleen	4.6	1.30	0.60
Tumor bearing	Serum	7.4	1.18	0.76
	Liver	4.0	1.06	0.23
	Spleen	5.2	1.61	0.76
	Tumor	5.4	4.08	1.00

Average of 5 mouse experiments
LDL: low density lipoprotein

tumor low density lipoprotein was different from that of normal, and also that of organs in tumor-bearing mice. The differences between low and high density lipoprotein of the tumor are clearly shown in Table 2. The phospholipid to protein ratio of spleen of tumor-bearing mice was greater than that of normal, and the ratio of tumor also greater than others.

TABLE 2 ANALYSIS OF TUMOR LIPOPROTEIN

	LDL	HDL
Protein	2.46	7.41
Total Lipid	13.50	3.49
Neutral Lipid	9.29	2.79
Phospholipid	5.26	0.71

unit=mg/ml Average of 5 mouse experiments
Lipoprotein suspension used for immunizing mice was analyzed.
HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein.

Ultracentrifugal flotation pattern is shown in Fig. 1. The flotation rate of tumor low density lipoprotein was Sf 5.9, and the molecular weight was presumed about 130×10^4 according to LINDGREN.

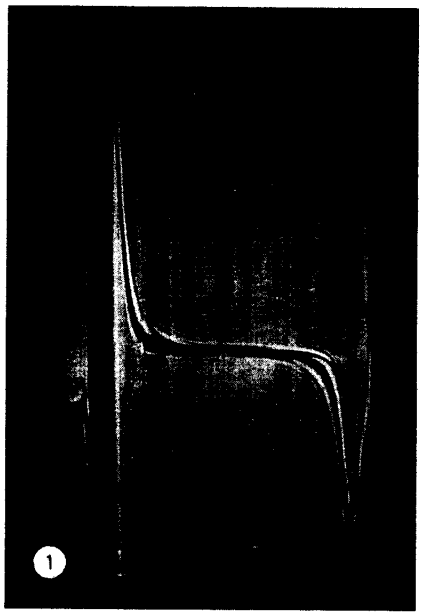


Fig. 1 Ultracentrifugal analysis of tumor low density lipoprotein. Flotation rate of tumor low density lipoprotein was analyzed in 1.063 density NaCl solution at 26°. The peak was observed at 30 min. of 51,2000 rpm, and the Sf was 5.9.

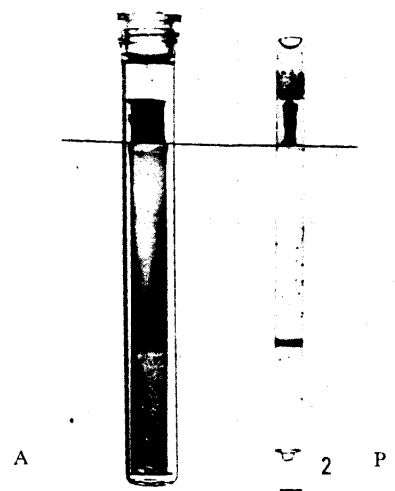
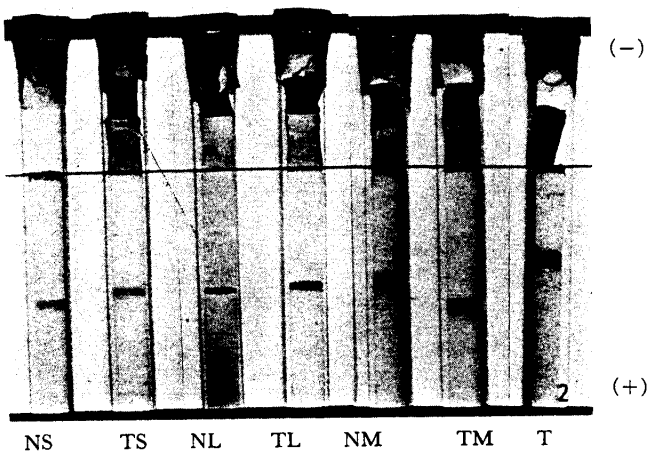


Fig. 2 1) Disc electrophoresis of low density lipoprotein.
A: stained by Amido black, P: prestained by Sudan black B.



2) Disc electrophoresis of low density lipoprotein in mice.
NS = normal mice serum, TS = serum of tumor-bearing mice, NL = low density lipoprotein from liver of normal mice, TL = low density lipoprotein from liver of tumor-bearing mice, NM = low density lipoprotein from spleen of normal mice, TM = low density lipoprotein from spleen of tumor-bearing mice, T = low density lipoprotein of Ehrlich ascites tumor.

Electrophoretic patterns were shown in Fig. 2. Low density lipoproein migrated as a single band by prestaining method, and the same mobility was observed with amido black stain. The electrophoretic mobility of low density lipoprotein of serum and liver in tumor-bearing mice were slower than normal ones, and that of spleen were faster than the others. Electrophoretic mobility was slowest in tumor tissue.

Antitumor Activity of Immune-serum: Antitumor activity was examined *in vitro* cultured JTC-11 cells. Antitumor activities of serum, supernatant of lymph nodes and spleen homogenate of immunized with low density lipoprotein are shown in Fig. 3. No significant inhibitory effect on tumor growth was observed in these of immunized with high density lipoprotein. Antitumor activity of immune-serum was most elevated at 25th day after immunization as seen in Fig. 4, and the antitumor activity was gradually decreased from 30th day or later. Fig. 5 illustrates the relations between antigenicity and lipoprotein concentration. The highest antigenicity was observed by immune-serum obtained from mice injected with 1 mg of protein concentration of tumor lipoprotein. Protein concentration above 1 mg was unable to examine, because mice administered aove 1 mg protein were almost died earlier than 25th day. The antitumor activity of immune-serum existed mainly in γ-globulin fraction separated by DEAE-Sephadex A-50 column as seen in Fig. 6.

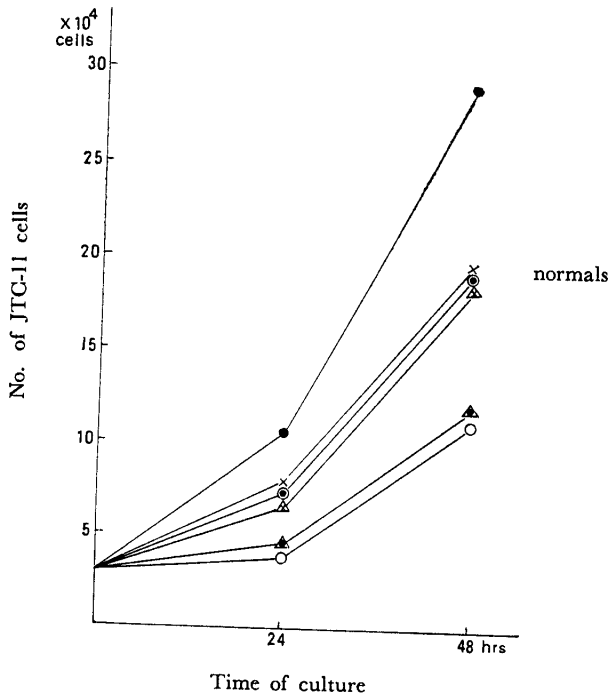


Fig. 3 Antitumor activity of immune-serum. Normal serum, and the supernatant of immunized lymph nodes and spleen homogenate, each 1 mg/ml protein concentration, were added for cell culture tubes.
●; control, ×; normal serum, ⊙; serum immunized with high density lipoprotein, ▲; supernatant of lymph nodes and spleen immunized with high density lipoprotein, △; supernatant of lymph nodes and spleen immunized with low density lipoprotein, ○; serum immunized with low density lipoprotein.

DISCUSSION

The cell culture technique to prove the tumor cytotoxicity was reported by MATUMOTO (18), that rabbit immune-serum produced by lipopolysaccharide was specific for the cultured cell growth. BJÖRKLUND (19) also reported thas the specific antigen of HeLa cell was probably existed in membrane structure lipoprotein.

In the present experiment, the antitumor activity of immune-serum produced by low density lipoprotein in tumor transplanted with JTC-11 cell was explored with *in vitro* cultured JTC-11 cells.

Tumor low density lipoprotein was especially antigenic, and high density lipoprotein no antigenic. This suggests that the tumor antigenicity might not be existed in the protein structure of tumor cell, but in the

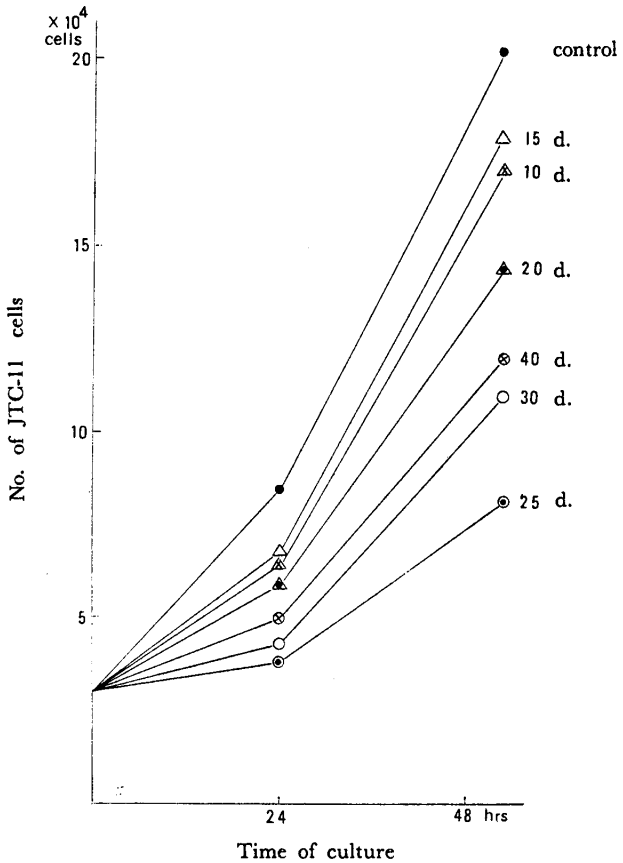


Fig. 4 Antitumor activity of γ-globulin fraction of immune-serum after immunization. Immune-serum was obtained on various days after immunization, and immuno-globulin was precipitated by addition of 50% ammonium sulfate. Each 1 mg/ml protein concentration was added in cell culture tube after dialysis. d = days after immunization.

lipid composition, especially in phospholipids of low density lipoprotein as illustrated in Table 1, because the phospholipid content and the electrophoretic mobility of tumor low density lipoprotein were different from others. SACKS *et al.* (20) reported that the electrophoretic mobility of human serum incubated with soya bean lipoprotein migrated faster than that of non-treated one. Therefore, the differences of electrophoretic mobility of low density lipoprotein between normal and tumor-bearing mice might be concerned with the phospholipid content.

The antitumor activity of immune-serum was most elevated at 25th day after immunization, and that of lymph nodes and spleen was at 10th

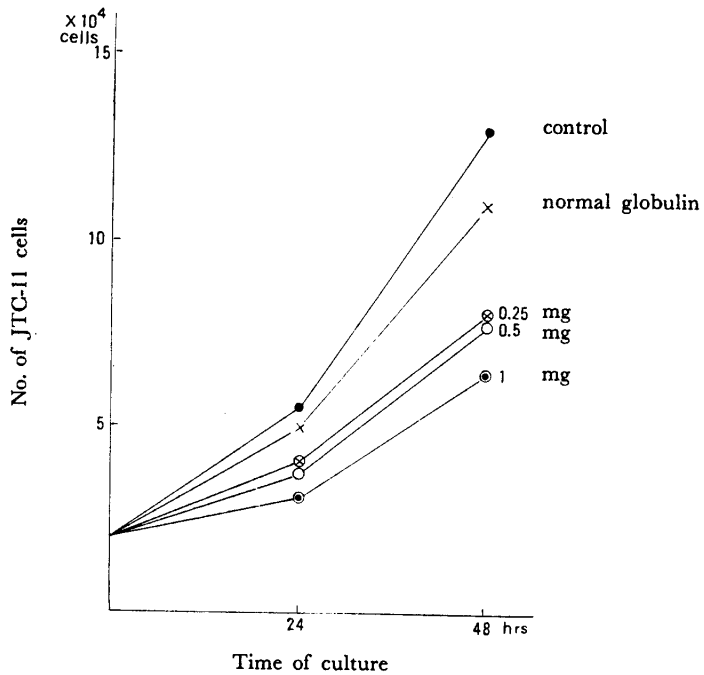


Fig. 5 Antigenicity of lipoprotein concentration. Immuno-globulin at 25th dayafter immunization, obtained by immunizing with 1 mg, 0.5 mg of protein concentration of tumor lipoprotein, was added in cell culture tube.

day as previously reported (4). These facts suggest that the antibody formation might be in lymph nodes and spleen in 10 days after immunization, and the immunoglobulin is gradually issued in serum.

In the present experiment, γ-globulin fraction of immune-serum acted as growth inhibitor *in vitro* cultured JTC-11 cells. HIRAI (21) reported that the γ-globulin of tumor transplanted rat was decreased, and that of spontaneously healing was increased.

In the present experiment, the possibility of antibody formation for malignant tumor was demonstrated by antigenicity originated from tumor low density lipoprotein.

CONCLUSION

A unique low density lipoprotein was obtained from the tumor transplanted with a cultured cell line of Ehrlich ascites tumor, JTC-11 cell. The tumor low density lipoprotein electrophoretically migrated as a single band, and the mobility was different from that of other organs. The

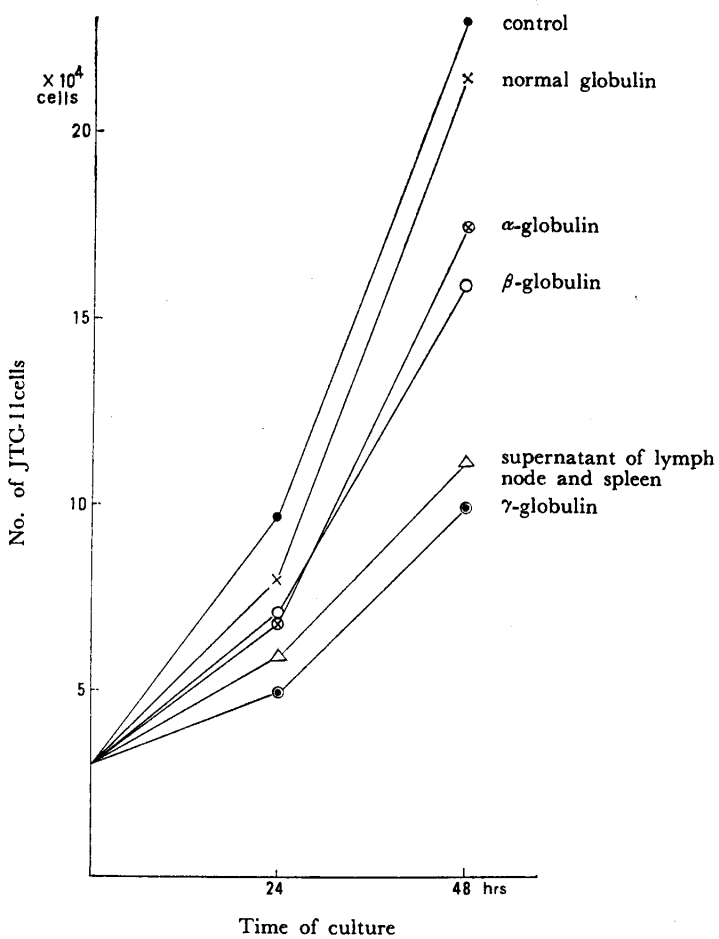


Fig. 6 Antitumor activity of immuno-globulin. α-, β- and γ-globulin fraction each 1 mg/ml protein concentration was added for cell culture tube.

chemical composition of lipid, cholesterol and phospholipids in tumor low density lipoprotein were characteristic. The flotation rate was Sf 5.9, and thus the molecular weight was estimated to be about 130×10^4.

The inhibitory effect on tumor growth of the immune serum was most elevated at 25th day after the intraperitoneal administration of tumor low density lipoprotein. The main fraction effective for inhibition of tumor growth existed in γ-globulin.

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