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# ISOLATION OF TUMOR DISTURBING FACTOR ON THE PROLIFERATION OF TUMOR CELLS IN HUMAN SERUM

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SUMMARY: The classified sediment with ethanol from sera of nude mice and humans showed a disturbing effect on L1210 cells in vitro and a lifesaving effect on L1210 cell-bearing mice in vivo. This factor was purified more than 2300-fold to a specific activity of approximately $1x10^5$ U/mg by ethanol classified precipitation, Sephadex G-200 gel filtration, DEAE cellulose ion exchange chromatography with Nacl and pH gradient aqueous solution, and preparative polyacrylamide gel electrophoresis.

INTRODUCTION: Current development of vital components in the physiological field of antitumor phenomena have involved studies in areas such as lymphokines(1-3), tumor necrosis factor(4-10) and humoral antibody(11). However,all such agents show a killing effect on tumor cells. Corwin et al(12) reported that the Kirsten sarcoma virus-transformed cell line,K3T3, when grown in media containing delipidized fetal calf serum, regained many of the properties of the untransformed parent cell. Such properties include morphology,adherence,saturation density and growth patterns. This suggests that there is possibly an effective factor in the delipidized serum which changes properties in a transformed cell. This report presents data indicating that it is possible that the tumor disturbing factor on the growth patterns is in the delipidized sera of the nude and human.

Such tumor disturbing factor(T.D.F.) was actually isolated in purified form from delipidized human serum.

Abbreviation: T.D.F.= Tumor disturbing factor

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MATERIAL AND METHODS:

Serum: Nude mice(BALB/c) were obtained from Sankyo Labo-Service Co.,Ltd.,Tokyo, and human serum was obtained from Tokyo Clinical Laboratory.

Bioassay for L1210 cells in tissure culture: The inhibition activity of all samples was bioassaied against L1210 cells in PRMI-1640 medium. Before testing,all samples were dialyzed against 1-3 changes of phosphate buffered solution. The units of inhibition activity were determined by the dilution analysis of samples and purified active factor as a standard. Initial density of $5x10^4$ cells/ml was used for L1210 cells. The number of cells living after 5 days in culture at 37°C were counted by the trypan blue dying method. Inhibition percent was calculated to compare the number of cells in a saturation density with and without sample.

Bioassay to determine the lifesaving effect on L1210 cell-bearing mice: The lifesaving effect was determined by measuring the survival term of L1210 cell-bearing mice and T/C value was calculated. Five BDF mice( ♂ 20g) were used per bioassay and L1210 cells were inoculated at $1 x10^6$ cells/mouse by interperitoneal injection.

Polyacrylamide gel electrophoresis: A ten-thirty μg of samples in Tris-glycinate buffered solution(pH8.3) was applied on a tube of 0.5x9, 12 and 24 cm(9 cm for purification, 12 cm for the degree of purity and 24 cm for molecular weight) of 7% polyacrylamide gel under 5 mA/tube, 3-6 hrs.. Active samples in polyacrylamide(pH 8.3) for 15 hrs. by electrophoresis. To determine the T.D.F. purity, the buffered polyacrylamide gels of pH 2.3, 4.3, 6.6, 8.0, 8.5, 9.5 and 10.0 were used in electrophoresis.

Chemicals: Tissure culture medium was purchased from the Japan Flow Lab.Inc.Tokyo and other chemicals used were purchased from Sigma or Merck.

RESULTS:

Disturbing effect of classified sediment on L1210 cells: The classified sediment with ethanol from sera of nude mice(BALB/c) and human was examined in the tissueculture of L1210 cells. The results of their saturation density on 5 day tissue culture are shown in Table 1.

Table 1. Disturbing effect of classified sediments with ethanol from sera of nude mice (BALB/c) or human on saturation density of L1210 cells on 5 day tissue culture

| | Ethanol | Saturation density[a] (cells/ml) | | | |
|---|---|---|---|---|---|
| | | Nude (BALB/c) content | | Human content | |
| | ( % ) | 1 mg/ml | 0.1 mg/ml | 1 mg/ml | 0.1 mg/ml |
| 1 | 0 - 30 | 2.9 | 3.2 | 3.0 | 3.1 |
| 2 | 30 - 40 | 2.8 | 2.9 | 3.1 | 3.1 |
| 3 | 40 - 45 | 1.9 | 2.9 | 2.8 | 3.2 |
| 4 | 45 - 50 | 0.9 | 1.6 | 0.9 | 1.8 |
| 5 | 50 - 60 | 1.8 | 2.7 | 2.1 | 2.8 |
| 6 | 60 - 70 | 1.8 | 2.2 | 1.9 | 3.0 |
| 7 | Sup. | 2.2 | 2.9 | 2.6 | 2.9 |

a) Values for triplicate experiments are given.

Initial cell density was $5 \times 10^4$ cells/ml

Table 2. Lifesaving effect of classified sediments with ethanol from sera of nude mice (BALB/c) or human on L1210 cell transplanted mice

| Ethanol | Nude mouse | | | Human | | |
|---|---|---|---|---|---|---|
| ( % ) | S.T.[a] (days) | ( ± S.D.)[b] | T/C[c] ( % ) | S.T. (days) | ( ± S.D.) | T/C ( % ) |
| 0 - 30 | 9.3 | 0.7 | 99 | 10.6 | 0.6 | 103 |
| 30 - 40 | 9.0 | 0.0 | 96 | 10.0 | 1.0 | 97 |
| 40 - 45 | 9.7 | 0.7 | 103 | 10.0 | 0.0 | 97 |
| 45 - 50 | 14.3 | 0.7 | 152 | 19.6 | 2.6 | 190 |
| 50 - 60 | 10.0 | 1.0 | 106 | 12.5 | 0.5 | 121 |
| 60 - 70 | 9.7 | 0.7 | 103 | 10.6 | 0.6 | 103 |
| Sup. | 9.7 | 0.7 | 103 | 11.0 | 0.0 | 107 |
| Control | 9.4 | 0.6 | | 10.3 | 0.7 | |

a) S.T. : Survival term (days after cell inoculation)
b) S.D. : Standard deviation
c) T/C : Test by control on survival term (days)

$1 \times 10^6$ L1210 cells were inoculated, and sample was injected (4 mg/body, at day 1,3,5,7 and 9) into 20 g $BDF_1$ mouse (five mice in a group) by i.p..

Among the samples No.4 showed the most effective disturbing action on proliferation of L1210 cells.

Lifesaving effect of classified sediment on L1210 cell-bearing mice: The lifesaving effect of the classified sediment with ethanol from sera of nude mice(BALB/c) and human on mice with L1210 cells is shown in Table 2. Results indicated that the No.4 sample has an active lifesaving effect *in vivo*, as it does *in vitro*.

Purification of active factor: All steps were conducted at 4°C unless otherwise indicated. A typical purification of T.D.F. was as follows (Table 3).

Acetone precipitation: Acetone, double the volume of serum, was added to the serum at -20°C. All the sediment was dried in vacuum after 1 hr. of precipitation at -20°C. Seventy-seven and four-tenths grams of sediment(43 U/mg) was obtained from 1 L of human serum.

Alcohol fractionation: Studies revealed that the greatest active fraction was found in the presence of 45 to 50% alcohol at 4°C. Eight and six-tenths grams of active fraction(208 U/mg) was obtained from the sampl(43 U/mg).

Table 3. Purification of T.D.F.

| Step | Weight | Protein ( % ) | U/mg | Total Units | Yield in each step ( % ) | Total yield ( % ) |
|---|---|---|---|---|---|---|
| Human serum | 1 L | | | | | |
| Acetone ppt. | 77.4 g | 60.8 | 43 | 3328200 | | 100 |
| Ethanol ppt. | 8.6 g | 84.3 | 208 | 1788800 | 53.7 | 53.7 |
| Sephadex G-200 | 1.57 g | 87.4 | 660 | 1036200 | 58.0 | 31.1 |
| DEAE A25(NaCl) | 196 mg | 90.6 | 1040 | 203840 | 19.7 | 6.1 |
| DEAE A50(NaCl) | 24.5 mg | 91.7 | 4139 | 101406 | 49.8 | 3.0 |
| DEAE A50(pH) | 3.5 mg | 93.5 | 10680 | 37380 | 37.2 | 1.1 |
| Electrophoresis | 57 μg | 99.3 | 100000 | 5700 | 56.2 | 0.62 |

Sephadex G-200 gel filtration chromatography: At this stage of the purification, the major contaminating protein species were in the M.W. range of 60,000 to 80,000 daltons as determined by SDS-polyacrylamide gel electrophoresis. Ten grams of material from the alcohol fraction was therefore chromatographed on a 3.5x60 cm Sephadex G-200 gel column equilibrated in 0.05M Tris-Hcl solution(pH 7.3,4°C). The T.D.F. containing fractions were pooled, dialyzed against distilled water and precipitated with acetone.

DEAE A-25 ion exchange cellulose chromatography with Nacl gradient: Five hundred mg of material from Sephadex G-200 gel filtration was chromatographed on a 2.1x40 cm DEAE A-25 ion exchange cellulose Nacl gradient(0-0.5M) in 0.05m Tris-Hcl,pH 7.3(4°C).

DEAE A-50 ion exchange cellulose chromatography with Nacl gradient: One hundred mg of material from the DEAE A-25 cellulose experiment was chromatographed on a 1.0x30 cm DEAE A-50 ion exchange cellulose with Nacl gradient(0-0.5M) in 0.1M Tris-Hcl,pH 7.3(4 C).

DEAE A-50 ion exchange cellulose chromatographed with pH gradient: Fifty mg of material from the above experiment was chromatographed on a 1.0x20 cm DEAE A-50 ion exchange cellulose with pH gradient(pH 5.0-8.0) in 0.1M Nacl aqueous solution.

Preparative polyacrylamide gel electrophoresis: A thirty μg of sample from the above experiment in Tris-glycinate buffered solution (pH 8.3) was applied to a tube(0.5x9 cm) of 7% polyacrylamide gel

under 5 mA/tube for 4 hrs.. Three protein bands showed in the gel tubes were collected, mixed with 7 % new polyacrylamide gel and again poured into tubes of the same gel condition. T.D.F. in the gel tube was eluted by electrophoresis to the buffered solution(pH 8.3) for 15 hrs.. The eluted sample was dialyzed in distilled water and precipitated with acetone.

Gel electrophoretic analysis of T.D.F.: To determine the degree of purity of the T.D.F., in a second procedure the sample was electrophoresed at pH 2.3, 4.3, 6.6, 8.0, 8.5, 9.5 and 10.0 instead of the two-dimensional gel system. The sensitivity of the system was enhanced by a silver staining procedure capable of detecting less than 1 ng of protein per $mm^2$ of gel(13). The band of T.D.F. in the gel at pH 2.3, 4.3 and 6.6 did not move at all by electrophoresis in a 3 mA/tube for 4 hrs.. Only a single band for T.D.F. showed in the 7% polyacrylamide gel at pH 8.0, 8.5, 9.5 and 10.0 by electrophoresis in a 3 mA/tube for 5 hrs., as shown in Table 4.

Color reaction: T.D.F. showed a positive reaction with the Ninhydrin reagent for protein. It also showed a positive reaction with anthrone, Molish and the periodic acid reagents for carbohydrate.

Molecular weight: To determine the M.W. of T.D.F., it was electrophoresed at pH 7.0 in 7 or 10% polyacrylamide gel (0.5x24 cm tube) in a 4 mA/tube for 7 hrs. at 4 C.. The presumed M.W. of T.D.F. was indi-

Table 4. The degree of purity for T.D.F. on electrophoresis

| No. | gel pH | charge time ( hr ) | Detection Rf | |
|---|---|---|---|---|
| 1 | 2.3 | 4 | 0 | |
| 2 | 4.3 | 4 | 0 | |
| 3 | 6.6 | 3 | 0 | |
| 4 | 8.0 | 5 | 0.33 | single band |
| 5 | 8.5 | 5 | 0.51 | single band |
| 6 | 9.5 | 5 | 0.74 | single band |
| 7 | 10.0 | 4 | 0.81 | single band |

7% polyacrylamide gel (0.5 x 12 cm),3 mA/tube charge, sample scale are 50 μg/tube or 100 μg/tube.

Table 5. SDS-polyacrylamide gel electrophoresis

| 10 % gel : 1st experiment | | | 7 % gel : 2nd experiment | | |
|---|---|---|---|---|---|
| sample | M.W. | Rf | sample | M.W. | Rf |
| T.D.F. | | 0.153 | Hexokinase(yeast) | 104,000 | 0.159 |
| Transferrin | 74,000 | 0.154 | T.D.F. | | 0.635 |
| Albumin(horse) | 70,000 | 0.185 | Transferrin | 74,000 | 0.639 |
| Albumin(egg) | 45,000 | 0.401 | Albumin(horse) | 70,000 | 0.714 |
| Trypsine | 23,300 | 0.716 | Albumin(human) | 69,000 | 0.738 |
| C ytochrome C | 15,600 | 0.906 | Albumin(calf) | 65,400 | 0.802 |

M.W. : Known molecular weight

cated as 74,100-74,300 daltons in the first experiment, and it was presumed as 74,100 daltons in the second experiment(Table 5).

DISCUSSION:

The scheme used to isolated T.D.F. is summarized in Table 3. In this particular purification, the starting material had a specific activity of approximately 43 U/mg, and the specific activity of the final product increased about 2,300-fold to a value of $1x10^5$ U/mg. The yield of T.D.F. was very low, being in the range of 0.1 to 1.0% of the starting material. The major loss of material occurred at the DEAE A-25 ion exchange cellulose chromatography step. Nonetheless,the purification yielded 57 Ag of active material from 1 L of human serum. There have been many reports of protein with M.W. in the range of 64,000-84,000 and in relation to human tumor or human serum, for example,differentation markers in human malignant melanoma(14), T cell factor(15), T lymphocyte I region antigen(16),viral polypeptides(17), dimer fibloblast interferon(18), T cell surface protein(19,20), thymus and T cell antibody(21), migration inhibitory factor(MIF)(22), surface protein of SV-40 transformed cell(23), thymocyte-activating factor(24), receptor for tumor-associated fetal antigens(25), receptor for IgG(26), human liver derived inhibitory protein(LIP)(27),human serum spreading factor(28) and B 73.1-defined antigen(29). It is surprising to find the same known compounds with the same M.W. value and the same physiological activity.

The activity of T.D.F. against sarcoma-180 cells on the proliferation of the tissue culture showed $1x10^5$ U/mg in the dilution method,

and the degree of intensity of this special activity was almost the same as the activity against L1210 cells. This active factor has a disturbing effect on the saturation density of proliferation in tumor cells, but showed no killing effect against tumor cells or normal cells from rat liver.

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REFERENCES:

1. Granger,R.A. and Williams,T.W. (1968) Nature 218,1253-1254.
2. Dumonde,D.C.,Wolstencroft,R.A.,Panayi,G.S.,Matthew,M.,Morely,J.and Howson,W.T. (1969) Nature 224,38-42.
3. Mani,R.N.Bryceson,A.D.M.,Wolstencroft,R.A. and Dumonde,D.C. (1969) Nature 224,43-44.
4. Carswell,E.A.,Old,L.J.,Kassel,R.L.,Green,S.,Fiore,N. and Williamson ,B. (1975) Proc.Natl.Acad.Sci.U.S.A. 72,3666-3670.
5. Matthews, N. and Watkins,J.F. (1978) Br.J.Cancer 38,302-309.
6. Matthews,N. (1978) Br.J.Cancer 38,310-315.
7. Ostrove,J.M.andGifford,G.E. (1979)Proc.Soc.Exp.Biol.Med. 160,354-358.
8. Helson,L.,Helson,C.andGreen,S. (1979) Exp.Cell Biol. 47,53-60.
9. Clark,I.A. (1979) Infect. Immunol. 24,319-325.
10. Haskill,S.,Ritter,F. and Becker,S. (1980) J. Immunol.125,454-458.
11. Lando,P.,Blomberg,F.,Berzins,K.and Perlman,P. (1980) Cancer Res. 40,1695-1698.
12. Corwin,L.M.,Humphrey,L.P. andShloss,J. (1977) Exp. Cell Res. 108, 341-347.
13. Switzer,R.C.III.,Merrill,C.R. andShifrin,S. (1979 )Anal. Biochem. 98,231-237.
14. Tai,T.,Eisenger,M.,Ogata,S. and Loyd,K.O. (1982) Cancer Res.42, 2773-2779.
15. Miyatani,S.,Hiramatsu,K.,Nakajima,P.B.,Owen,F.L. and Tada,T.(1983) Proc.Natl.Acad.Sci.U.S.A. 80,6336-6340.
16. Hullett,D.A. and Hayes,C.E. (1982) Ir genes, 5,169-173.
17. Waner,J.L.,Kong,N. and Biano,S. (1983) Infect.Immunol.41,1084-1088.
18. Pastka,S.,Kelder,B.,Familletti,P.C.,Moschera,J.A.,Crowl,R. and Kempner,E.S. (1983) J.Biol.Chem.258,9706-9709.
19. Chin,Y.H.,Carey,G.D. and Woodruff,J.J.(1983) J.Immunol. 131,1368-1374.
20. ConeR.E.,Rosenstein,R.W.,Tite,J.,Ptak,W. and Gershon,R.K.(1983) J. Immunol.130,2083-2087.
21. Ishii,Y.,Kon,S.,Takei,T.,Fujimoto,J. and Kikuchi,K. (1983) Clin. Exp.Immunol.53,31-40.
22. Remold,H.G.,Mednis,A.,Kawaguchi,T.,Bersch,N. and Golde,D.W.(1983) Cell Immunol.78,305-313.
23. Rose,T.M. and Weil,R. (1983) Int.J.Cancer 31,639-648.
24. Grabner,G.,Luger,T.A.,Luger,B.M.,Smolin,G.and Ch,J.O. (1983) Invest. œhthalmol.Visual.Sci.24,589-595.
25. Evans,D.L.,Hooper,C.J.,Wiggins,J.B.,Shehee,W.R.,Hale,A.H.,Hogan,K.T. and Johnson,K.D. (1982) Oncodev.Biol.Med.3,365-378.
26. Anderson,C.L. (1982) J.Exp.Med.156,1794-1806.
27. Grol,M.and Schumacher,K.(1983) J.Immunol.130,323-326.
28. Barnes,D.W.,Silnutzer,J.,See,C. and Schaffer,M. (1983) Proc.Natl. Acad.Sci.U.S.A. 80,1362-1366.
29. Perussia,B.,Acuto,O.,Terhorst,C.,Faust,J.,Lazarus,R.,Fanning,V.and Trinchieri,G. (1983) J.Immunol.130,2142-2148.