

SERUM FACTORS THAT SUPPRESS CYTOTOXIC EFFECT OF METHOTREXATE

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Aim: To study the phenomenon that human erythroid leukemia K-562 cells are more sensitive to cytotoxic effect of antimetabolites when cultured in a serum-free medium than in a conventional medium containing fetal calf serum (FCS). **Methods:** Cytotoxic effects of methotrexate, azaserine and 5-fluorouracil were estimated by accessing the lactate dehydrogenase (LDH) activity of viable tumor cells. Proteins of FCS were separated using two-dimensional electrophoresis followed by mass spectrometry analysis. **Results:** Addition of 10% FCS attenuated anti-tumor activity of methotrexate and azaserine against K-562 cells compared with serum-free medium. Such an activity of FCS was different for each serum lot. Comparison of the proteins in active serum lot with those in not active one using two-dimensional electrophoresis showed that in the active serum there were proteins 150 kDa, which were absent in the not active serum lot. Mass spectrometry indicated that all those proteins had the amino acid sequence of albumin. Sera of one healthy volunteer and two patients with thyroid cancer also attenuated the activity of the agent. **Conclusion:** Several lots of FCS and human serum demonstrated the ability to attenuate the cytotoxic effect of methotrexate *in vitro*, possibly due to the formation of albumin dimers/MTX complexes.

Key Words: serum, methotrexate, azaserine, K-562, anti-tumor activity.

Antimetabolites, e. g., methotrexate (MTX), cytosine arabinoside (Ara-C) and 5-fluorouracil (5-FU), are widely used drugs in anti-tumor therapy. However, the currently used antimetabolites have not displayed satisfactory results in the clinical treatment of solid tumors and leukemia. Originally antimetabolites were thought to inhibit the activities of enzymes for *de novo* pathways in nucleic acid biosynthesis. However, in cancer cells the activities of enzymes for salvage pathway of nucleic acid biosynthesis are also high. So, researchers proposed to combine inhibitors of key enzymes for *de novo* pathways of nucleic acid biosynthesis with blockers of the salvage pathway [1, 2].

Recently, we have observed the phenomenon that the anti-tumor activity of azaserine, one of the antimetabolites used for *in vitro* studies of human erythrogenic leukemia cell line K-562, was altered by different culture medium. The anti-tumor activity was higher in a serum-free medium Cosmedium-001 than in another serum-free medium –ASF 104, or modified minimum essential medium (MEM) with 10% fetal calf serum (FCS).

MATERIALS AND METHODS

Cells, medium and FCS. A human erythroid leukemia cell line K-562 was maintained in 10% FCS modified Eagle's minimum essential medium (MEM). Modified MEM was composed of 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 25 mM HEPES and MEM. Two serum-free media, Cosmedium-001 and

ASF 104 were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan) and AJINOMOTO Co., Ltd. (Tokyo, Japan), respectively. Two lots (SFB30-1674 & 1663) of FCS were purchased from Equitec-Bio, Inc. (TX, USA), and inactivated by incubation at 56 °C for 30 min. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

Antimetabolites. Azaserine, 5-FU, MTX, Ara-C and trimethoprim were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Drugs were used at following concentrations: 10–40 μ M, 500–2000 ng/ml, 0.025–0.1 μ M, 25–100 μ g/ml and 0.25–1 mM, respectively.

Human serum albumin. Albumin (25%) Cutter (D419) and albumin from human serum (ALF0020) were purchased from Bayer in Japan (Osaka, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively.

Ultrafiltration. FCS or medium was filtered with the use of Amicon centrifugal filter devices, YM-30 (nominal molecular weight: > 30 kDa) and YM-100 (nominal molecular weight: > 100 kDa) (Millipore Corporation, Bedford, MA, USA). Preliminary experiments showed that retentates of ultrafiltration with YM-30 and -100 contained molecules with molecular weight 30 kDa and 100 kDa, respectively. Both filtrate and retentate were reconstituted with the starting medium.

Estimation of anti-tumor activity. Human leukemic cells, mainly K-562 cells (5×10^3 /well) were incubated with several factors in 96-well round-bottomed microtiter plates, each well containing 200 μ l of medium at 37 °C for 48 h. Then, the number of viable cells was estimated by measuring the activity of released LDH from viable cells using a kit, CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, USA).

Two-dimensional electrophoresis and mass spectrometric analysis of proteins. In order to de-

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Abbreviations used: 5-FU – 5-fluorouracil; ACN – acetonitrile; Ara-C – cytosine arabinoside; BSA – bovine serum albumin; FCS – fetal calf serum; HSA – human serum albumin; LDH – lactate dehydrogenase; MEM – minimum essential medium; MTX – methotrexate; TFA – trifluoroacetic acid.

fect factors, responsible for the effect of FCS, we have used the technical services of Towa Kankyo Kagaku Co. Ltd. (Hiroshima, Japan). Two lots of FCS (active and no activat) were separated by 2-dimensional electrophoresis, and analyzed by mass spectrometry. Isoelectric focusing (IEF) was performed using Immobiline dry strip pH 4–7 (GE Healthcare UK Ltd.) and Ampholine pH 3.5–10. Solubilized protein (100 µg) was loaded onto a strip. SDS-PAGE was performed using 9–18% gradient gels. Protein spots were detected by SYPRO Ruby staining (Invitrogen, CA, USA).

Protein spots were excised from the 2-D gels, washed twice with Milli-Q water, and dehydrated in 100% acetonitrile (ACN) until they turned opaque white. The spots were then dried in a vacuum centrifuge, and subsequently rehydrated in 10 µl of digestion solution consisting of 50 mM NH₄HCO₃, 5 mM CaCl₂, 0.01 µg/µl modified sequence-grade trypsin (Promega). After 16 h incubation at 37 °C the digestion was terminated by adding 10 µl of 5% trifluoroacetic acid (TFA). Peptides were extracted 3 times for 20 min with 50 µl of 5% TFA, 50% ACN, and the extracts were pooled and dried in a vacuum centrifuge. The dried materials were resuspended in 10 µl of 0.1% TFA. To remove excess salts from the extracts, solid-phase extraction was performed using C18 ZipTip (Millipore) according to the manufacturer's instruction. Peptides were eluted from ZipTip by 3 µl of 50% ACN, 0.1% TFA and 1 µl of the eluants were spotted onto a target plate. Then, the spots on the target plate were immediately mixed with 0.5 µl of a matrix solution containing 0.3 mg/ml α-cyano-hydroxycinnamic acid, 33% acetone, 66% ethanol, and were completely air-dried at room temperature. MS and MS/MS spectra were obtained using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics). An external peptide mixture was used to calibrate the instrument. Identification of proteins was carried out using the MASCOT software (Matrix Science) and de novo sequencing.

Albumin depletion. Depletion of albumin from FCS was performed with Vivapure® Anti-HSA Kit for human albumin depletion (Sartorius Biotech GmbH, Goettingen, Germany), which is based on an antibody to human serum albumin (HSA) or Montage™ Albumin Deplete Kit (Millipore Corporation, Bedford, MA, USA), which is based on an affinity resin, respectively. In case of Montage™ Albumin Deplete Kit, albumin depletion (more than 50%) was confirmed using SDS-PAGE. Both methods require buffer to deplete albumin from FCS. So the buffer was removed using a centrifugal concentrator, Apollo® 7 ml (Orbital Biosciences, LLC, Topsfield, MA, USA), and the obtained albumin-depleted FCS was reconstituted with PBS.

Statistics. Difference between 2 groups was evaluated using two-tailed Student's *t*-test or Welch's test. *P* values of 5 or less% were considered statistically significant.

RESULTS

Cytotoxic effect of antimetabolites on K-562 cells. Fig. 1, *a, b* shows that K-562 cells were

more sensitive to azaserine and MTX when cultured in Cosmedium-001 than in ASF 104 or 10% FCS modified MEM. Anti-tumor activity of other analyzed antimetabolites (5-FU, Ara-C and trimethoprim) was not affected by culture media (Fig. 1, *c–e*).

Effect of FCS on anti-tumor activities of anti-metabolites against K-562 cells cultured in Cosmedium-001. In order to examine why anti-tumor activities of MTX and azaserine were attenuated when K-562 cells were cultured in 10% FCS modified MEM, cells were cultured in Cosmedium-001 with 10% FCS. It was shown that anti-tumor activity of 5-FU was not altered by this medium. However, the anti-tumor activities of MTX and azaserine were attenuated.

Suppressive effect of different FCS lots on the anti-tumor activity of MTX. Fig. 3 shows that 10% FCS of lot No. 1674, but not lot No. 1663 attenuated the anti-tumor activity of MTX. Lot No. 1674 showed the highest activity among other tested lots of FCS, and the effect of lot No. 1664 was minimal (data not shown).

Molecular weights of the FCS molecules that suppress the anti-tumor activity of MTX. Ultrafiltration showed that FCS molecules 100 kDa from lot No. 1674 attenuated the cytotoxic effect of MTX (Fig. 4). However, a preliminary experiments revealed that this fraction actually contained molecules with approximate weight of 64.2 kDa (data not shown).

Two-dimensional electrophoresis of both lots of FCS. Fig. 5, *a, b* shows that the FCS (lot No. 1674) contained proteins with molecular weight of 150 kDa (*a*), which were absent in the FCS (lot No. 1663) (*b*). Mass spectrometry showed that those proteins had the same amino acid sequence as albumin.

Effect of albumin depletion on the suppressive activity of the FCS. Fig. 6 shows that depleting albumin from FCS (lot No. 1674) with 2 different methods (antibodies and affinity resin) attenuated its suppressive effect on anti-tumor activity of MTX.

Effect of human sera on the anti-tumor activity of MTX. Fig. 7 shows that sera of one female healthy volunteer and two female patients with thyroid cancer at a concentration of 5% completely abrogated the cytotoxic effect of MTX against K-562 cells cultured in Cosmedium-001 and that the activity of individual serum was different at concentrations of 0.05–0.5%.

Effects of HSA on the anti-tumor activity of MTX. It is well-known that the concentration of human serum albumin (HSA) in serum is 30–40 mg/ml. Five percent of human serum (1.5–2 mg/ml HSA) attenuated the anti-tumor activity of MTX (Fig. 7). So, we decided to test the ability of two HSA preparations at the concentration of 5 mg/ml, produced by different manufacturers, to suppress the anti-tumor activity of MTX (Fig. 8, *a*). One of the HSA preparations (Wako), lost its activity at a concentration of 10 mg/ml. A bovine serum albumin did not exert such an activity (data not shown). SDS-PAGE shows that not the BSA solution but the HSA (Wako) contained proteins with molecular weight of approximately 200 kDa (Fig. 8, *b*).

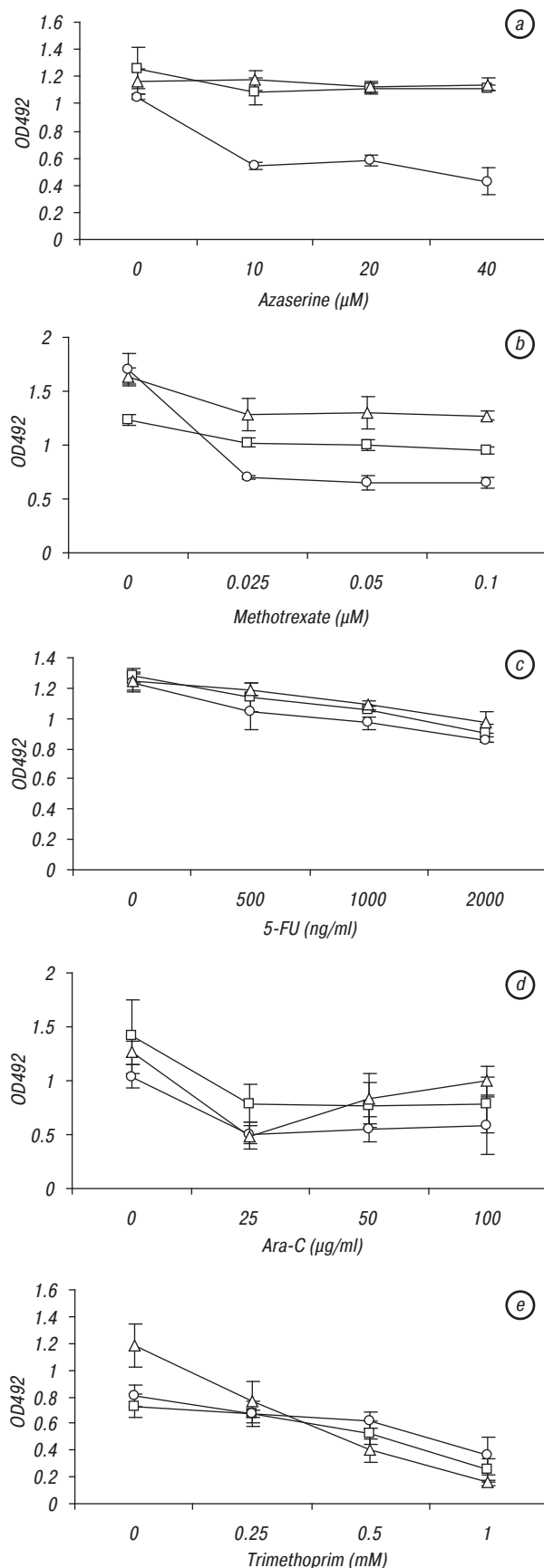


Fig. 1. Cytotoxic effect of antimetabolites on K-562 cells cultured in 3 different media. K-562 cells were incubated with the indicated concentration of antimetabolite, azaserine (a), MTX (b), 5-FU (c), Ara-C (d) and trimethoprim (e) in Cosmedium-001 (○), ASF 104 (△) or 10% FCS modified MEM (◻) at 37 °C for 48 h. Vertical bars represent standard deviation (SD) of 4 determinants

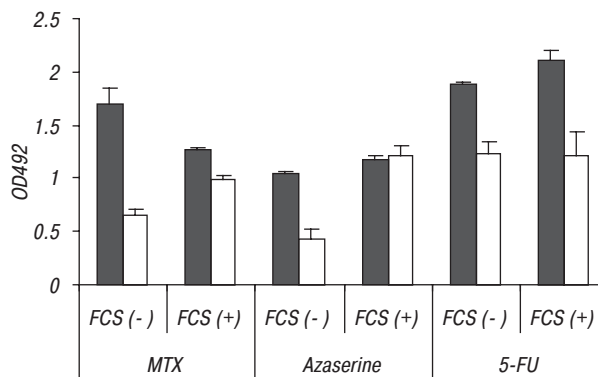


Fig. 2. Effect of FCS on anti-tumor activities of antimetabolites against K-562 cells cultured in Cosmedium-001. K-562 cells were incubated with (◻) or without (■) 0.1 μM MTX, 40 μM azaserine or 2000 ng/ml 5-FU in Cosmedium-001 containing 10% FCS < FCS (+) > or without FCS < FCS (-) > at 37 °C for 48 h. Vertical bars represent SD of 4 determinants

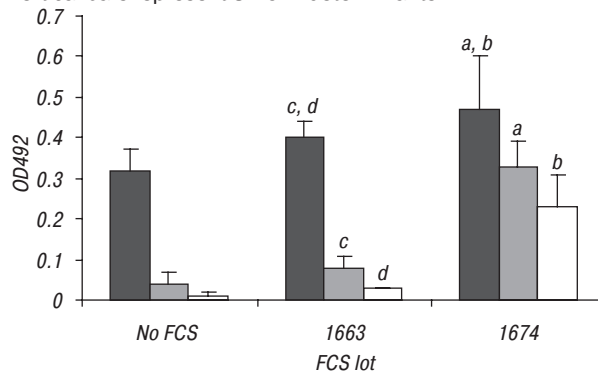


Fig. 3. Difference between two lots of FCS in their suppressive effect on the anti-tumor activity of MTX. K-562 cells were incubated without (■), or with 0.05 μM (◻) or 0.1 μM (◻) of MTX in Cosmedium-001 in the presence of 10% FCS (lot No. 1663 & 1674) or with no FCS at 37 °C for 48 h. Vertical bars represent SD of 4 determinants. ^aNot significant (NS), ^b*p* < 0.05, ^c^d*p* < 0.001.

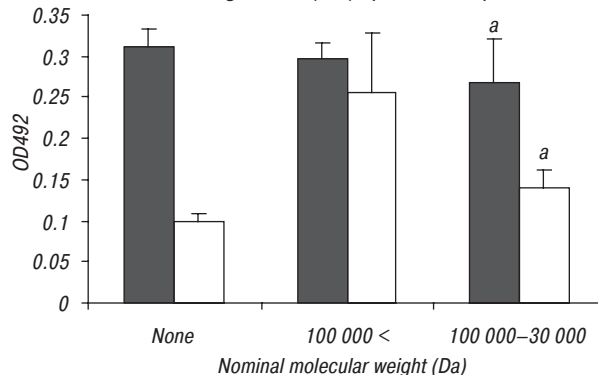


Fig. 4. The molecular weights of the molecules in the FCS that suppressed the anti-tumor activity of MTX. K-562 cells were incubated with (◻) or without (■) 0.1 μM MTX in 180 μl of Cosmedium-001 and 20 μl of the indicated FCS fraction at 37 °C for 48 h. Vertical bars represent SD of 4 determinants. ^a*p* < 0.01.

Effect of pre- or simultaneous treatment of K-562 cells with the FCS on the cytotoxicity of MTX. Fig. 9 shows that pre-incubation of K-562 cells with 10% FCS for 48 h did not attenuate their sensitivity to MTX, but only the simultaneous incubation of K-562 cells with 10% FCS and MTX had such effect.

Interaction between MTX and the suppressive molecules in the FCS. MTX (0.2 μM) and FCS (lot No. 1674) (20%) were incubated at 37 °C for 24 h. Their concentrations were 2 times higher than those in

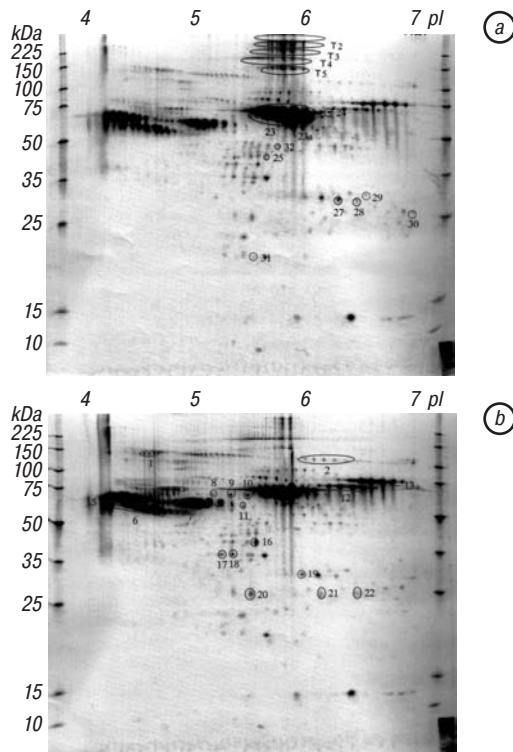


Fig. 5. Two-dimensional electrophoresis of two lots of FCS. The active FCS (No. 1674) (a) and the not active FCS (No. 1663) (b). Spots indicated by circles were specific in each FCS

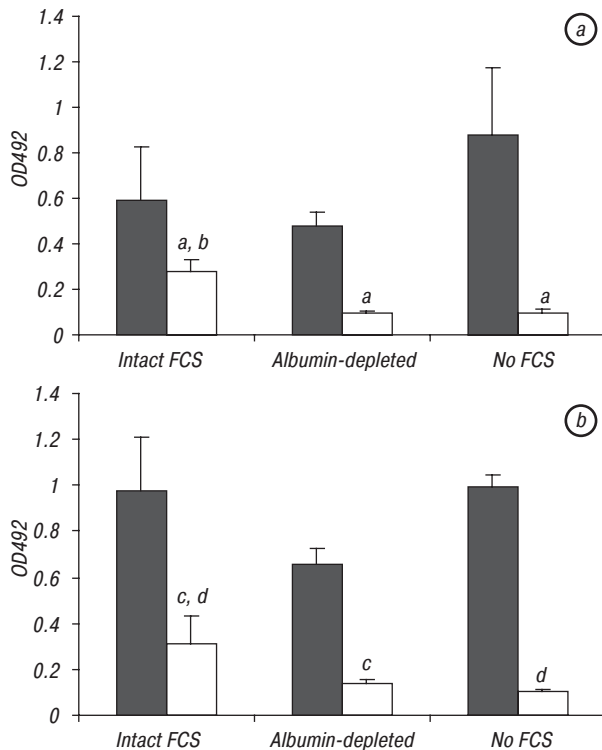


Fig. 6. Effect of albumin depletion on the suppressive activity of the FCS. K-562 cells were incubated with indicated fractions (10%) in the presence (□) or absence (■) of 0.1 μ M MTX in Cosmedium-001 at 37 °C for 48 h. Albumin depletion: Vivapure® Anti-HSA Kit (a), Montage Albumin Depletion Kit (b). Vertical bars represent SD of 4 determinants. * $p < 0.01$, ^b $p < 0.001$, ^c $p < 0.05$ (one-tailed), ^d $p < 0.05$.

the other experiments since they were finally diluted to 0.1 μ M (MTX) and 10% (FCS). Upon filtration by YM-100 filter, the anti-tumor activities of both filtrates and retentates were examined. Fig. 10 shows that the

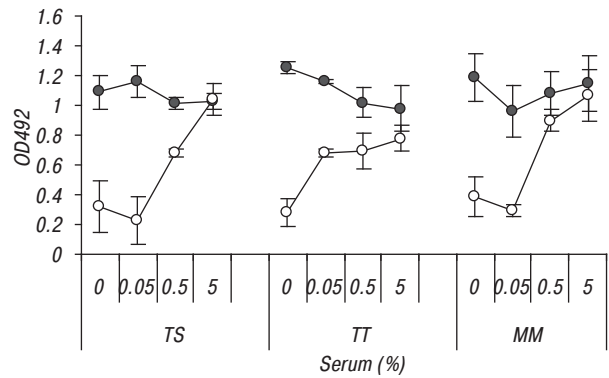


Fig. 7. Effect of human sera on the anti-tumor activity of MTX. K-562 cells were incubated with (○) or without (●) 0.1 μ M MTX in Cosmedium-001 containing the indicated concentrations of individual human serum at 37 °C for 48 h. TS, TT and MM represent initials of volunteers. Vertical bars represent SD of 4 determinants.

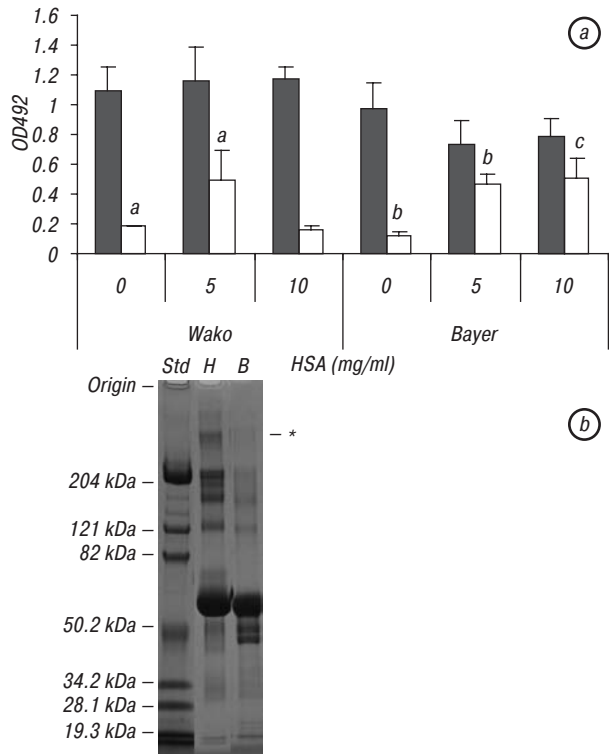


Fig. 8. Effect of HSA on the anti-tumor activity of MTX. K-562 cells were incubated with the indicated concentrations of HSA produced by two manufacturers, Wako and Bayer, in the presence (□) or absence (■) of 0.1 μ M MTX in Cosmedium-001 at 37 °C for 48 h (a). Vertical bars represent SD of 4 determinants. * $p < 0.05$, ^a $p < 0.05$, ^b $p < 0.001$, ^c $p < 0.05$. Wako's HSA (H) and BSA (B) were analyzed by SDS-PAGE (b). *Shows that HSA had indicated band, but BSA did not.

filtrate of MTX without the FCS exerted its anti-tumor activity. On the other hand, the filtrate of MTX incubated with the FCS did not exert its anti-tumor activity. Each retentate of MTX incubated with or without the FCS exerted weak anti-tumor activity to the same degree.

DISCUSSION

Many authors reported that albumin functions as a carrier for a variety of molecules. Albumin is the principle carrier of fatty acids [3–5], bilirubin [4, 5], metals including zinc [5, 6], hormones [7] and several important classes of drugs, e. g., calcium blockers, diuretics and non-steroidal anti-inflammatory drugs

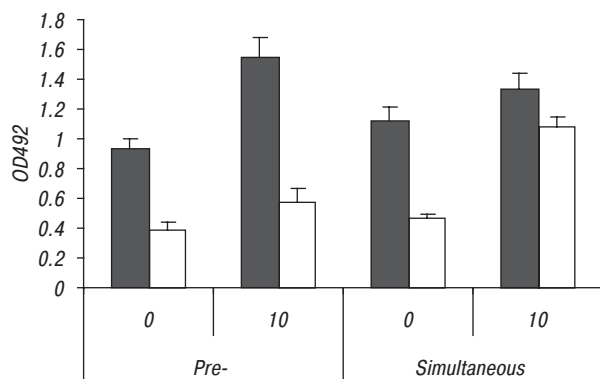


Fig. 9. Effect of pre- or simultaneous treatment of K-562 cells with the FCS on the anti-tumor activity of MTX. K-562 cells (2×10^5 /flask) were incubated with (10%) or without FCS in 3 ml of Cosmedium-001 at 37 °C for 48 h. After washing the cells twice with 3 ml of PBS, they were incubated (5×10^3 /well) with (□) or without (■) 0.1 μM MTX in 200 μl Cosmedium-001 at 37 °C for 48 h (Pre-). Alternatively, K-562 cells were incubated with (□) or without (■) 0.1 μM MTX in the presence (10%) or absence of FCS at 37 °C for 48 h (simultaneous). Vertical bars represent SD of 4 determinants

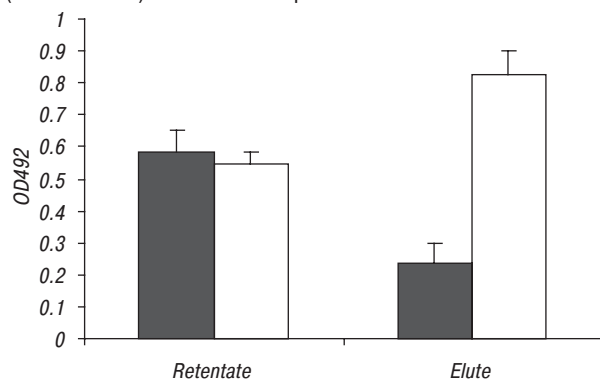


Fig. 10. Interaction between MTX and the suppressive molecules in the FCS. MTX (0.2 μM) was incubated with (□) or without (■) 20% FCS in 500 μl Cosmedium-001 at 37 °C for 24 h. The media were ultrafiltered with a YM-100 filter, and the filtrate and the retentate were collected followed by reconstitution with Cosmedium-001. K-562 cells were incubated in 100 μl Cosmedium-001 with 100 μl of the filtrate or the retentate at 37 °C for 48 h. Vertical bars represent SD of 4 determinants

[5]. All these authors consider albumin as a carrier molecule, especially Kratz [4] and his colleagues [8]. The present study suggested that proteins with high molecular weight derived from albumin (possibly its dimers or trimers) could bind MTX, and suppress its anti-tumor activity.

K-562 cells cultured in Cosmedium-001 were more sensitive to cytotoxic effect of azaserine and MTX than cells cultured in ASF 104 and 10% FCS modified MEM. The cytotoxic effect of other antimetabolites, 5-FU, Ara-C and trimethoprim against K-562 cells was not altered by culture media. When some lots of FCS were added to Cosmedium-001 at a concentration of 10%, MTX and azaserine exerted low anti-tumor activities to K-562 cells as compared with Cosmedium-001 alone.

This finding suggests that some factors in the FCS were responsible for suppressing the anti-tumor activities of both drugs. Lower anti-tumor activities of MTX and azaserine to K-562 cells cultured in ASF 104 than in Cosmedium-001, remain to be clarified.

The two-dimensional electrophoresis showed that the active FCS contained proteins with molecular weights 150 kDa, which were absent in the not active FCS lot. Mass spectrometry analysis revealed that those proteins had the same amino acid sequence as albumin. It seems that these proteins with high molecular weight are complexes of albumin. When albumin was depleted from the active FCS by means of 2 different methods (albumin binding affinity resin and anti-human serum albumin antibody), its suppressive activity was clearly attenuated. All tested human sera (one healthy volunteer and two patients with thyroid cancer) suppressed the anti-tumor activity of MTX against K-562 cells cultured in Cosmedium-001. Most of HSA samples are expected to exert such an activity. In fact, 2 tested commercially available HSA preparations exerted the activity. In any case, the activity of a larger number of human sera remains to be examined.

We hypothesized that the FCS did not resolve MTX chemically, but bound it. We suppose that possibly such MTX-binding molecules are complexes of albumin.

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