Elucidation of the Nature of the Murine Oncogenic Virus Inhibitor Isolated from JLS-V5 Cell Line (36044)

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Jasmin *et al.* reported the isolation of a substance from the supernatant fluid of cultures of a mouse spleen-thymus cell line chronically infected with Rauscher leukemia virus (JLS–V5) which markedly inhibited Friend leukemia virus (FLV) leukemogenic activity *in vivo* (1, 2). More recently, Chermann, *et al.* have shown that this substance inhibits Moloney murine sarcoma virus (M–MSV) focus-forming activity *in vitro* (3). This inhibitor, silicotungstic acid-treated supernatant of JLS–V5 cells (STAS), has been shown not to be interferon nor capable of inducing synthesis of interferon *in vitro* or *in vivo* (3). In addition, inhibitory substances capable of inhibiting murine leukemia and murine sarcoma viruses have been isolated from mouse spleen and bovine thymus by a procedure similar to that employed for the preparation of STAS (4).

This report concerns the quantitative and temporal aspects of STAS-induced inhibition of MLV replication *in vitro* as well as evidence which implicates silicotungstic acid as the component of STAS responsible for its inhibition of MLV replication *in vitro*.

Materials and Methods. Tissue culture cell lines. A cell line established from the bone marrow of Balb/c mice (JLS–V9), and the JLS–V5 cell line were kindly provided by Dr. Mayyasi, John L. Smith Memorial Institute for Cancer Research, Charles Pfizer and Co., Inc., Maywood, New Jersey. Mouse embryo fibroblast cells (NIH Swiss) were purchased from Microbiological Associates, Inc., Bethesda, Maryland, while the XC cell line was kindly provided by Dr. Rowe, National Institutes of Health, Bethesda, Maryland.

All cell cultures were grown on Eagle's minimum essential medium supplemented with 1% glutamine (200 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (Gibco, Inc., Grand Island, New York). Cells were removed from both glass and plastic surfaces by using 0.25% trypsin after 3 washes with phosphate-buffered saline (PBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Virus. Moloney leukemia virus (MLV) was kindly provided by Dr. Hercules, Microbiological Associates, Inc., Bethesda, Maryland. The virus was in its 42nd passage in NIH Swiss secondary mouse embryo fibroblast (MEF) cells. A virus pool was prepared from this stock MLV in secondary MEF cells according to the method described by Hartley *et al.* (5). This virus pool was shown to have a titer of $10^{6.7}$ plaque forming unit (PFU)/ml when titrated in MEF cells by the XC plaque assay procedure described by Rowe *et al.* (6).

XC plaque assay. The XC plaque assay for murine leukemia virus employed in these studies was identical to the procedure described by Rowe *et al.* (6).

Preparation of inhibitor. Silicotungstic acid-treated supernatant fluid from JLS–V5 cultures (STAS) was prepared according to the procedure described by Jasmin *et al.* (1). The silicotungstic acid (STA) used was either purchased from Fisher Scientific Co., or prepared by the method of Lindberg and Ernster (7).

Preparation of "mock" inhibitor. Preparations in which the starting material was not supernatant fluid from JLS–V5 cultures were prepared according to the above procedure for JLS–V5 (STAS). "Mock" preparations were prepared from: (i) Supernatant fluid from JLS–V9 cell cultures; (ii) Fresh tissue culture medium (JLS–V5 growth medium); (iii) Fetal calf serum; and (iv) Normal BALB/c

TABLE I. *In Vitro* Inhibition of MLV Replication by Preparations of JLS–V5 STAS as Measured by XC Plaque Reduction.

Source	MEF cell cultures treated with[a]	No. of culture plates/group	Av. no. of XC plaques/plate
—	MLV[b]	5	TNTC[e]
JLS-V5 (Jasmin)	STAS[c]	3	0
" "	STAS + MLV[d]	4	44
JLS-V5 (Ebert)	STAS	3	0
" "	STAS + MLV	4	8

[a] Plastic culture plates (60 mm) were seeded with 3.5 × 10^5 MSF cells/plate. Cultures were employed after 24 hr incubation at 37° in 5% CO_2.

[b] Cultures infected with 1000 ID_{50} of MLV/0.4 ml.

[c] Cultures treated with 1000 μg of STAS in 4.0 ml of growth medium.

[d] Cultures infected with 1000 ID_{50} of MLV/0.4 ml and treated with 1000 μg of STAS/4.0 ml at the time of infection.

[e] TNTC—too numerous to count.

mouse plasma. In addition, STA neutralized with NaOH and diluted to a final concentration of 0.01 *N* served as a "mock" inhibitor preparation. When neutralized 0.01 *N* STA (7) was dialyzed and lyophilized, a very small quantity of undetermined material could be recovered.

Additional "mock" inhibitor was prepared utilizing physiologic saline, instead of biological, fluids. Saline was treated with polyethylene glycol (PEG) as described by Jasmin *et al.* (1). The resultant supernatant solution was treated with 0.02 *N* STA, 0.02 *N* sodium silicate (Na_2SiO_3) (pH 5), or 0.02 *N* sodium tungstate (Na_2WO_4) (pH 5) and then centrifuged. The pH was adjusted to 1.0 and held overnight. The supernatant solutions were then processed as described earlier. One sample of saline was run through the inhibitor isolation procedure omitting the PEG treatment.

Inhibitor prepared without STA. Supernatant fluid from JLS–V5 cultures was treated with PEG as described by Jasmin *et al.* (1). However, instead of precipitating proteins with STA, other protein-precipitating procedures were substituted. Aliquots of virus-free supernatant cultures were treated with $ZnSO_4$ (1% final concentration), 5% trichloracetic acid (TCA), 5% perchloric acid (PCA), 80% ethanol, or 60% saturated $(NH_4)_2SO_4$. A sample diluted 1:1 with 0.02 *N* STA served as a positive control. Each mixture was immediately centrifuged at 10,000*g* for 20 min; the supernatant fluid was decanted and this fluid was held overnight at 4°. The next morning the pH of each supernatant solution was adjusted at 7.0. Any additional precipitate that formed, was removed by centrifugation and the supernatant solutions were put into washed dialysis bags. The preparations were dialyzed against cold distilled water for 4 days with 3–4 changes of water daily in the cold room (4°). The solutions in the bags were removed and then lyophilized. All inhibitor preparations were shown to be free of the precipitating agents used in their preparation.

Results. Chermann *et al.* (3) reported the inhibitory effects of STAS on *in vitro* focus-forming activity of M–MSV. Results in Table I show that STAS prepared by Dr. Jasmin and by our own laboratory (Ebert) was very effective in inhibiting *in vitro* replication of MLV in MEF cells. Both STAS preparations were shown to significantly inhibit MLV plaque formation *in vitro*. Subsequently, the STAS preparation of Ebert was employed in all the STAS experiments described in this report.

To determine the smallest quantity of STAS capable of reducing MLV-induced plaque formation by 50%, MEF cultures were treated with various concentrations of STAS (1000 μg—0.5 μg) at the time of infection with 50 PFU of MLV. As shown in Table II, treatment of MEF cultures with as little as 25 μg of STAS resulted in a 52% reduction

TABLE II. The Effect of Various Concentrations of JLS–V5 STAS on Replication of MLV *in Vitro*.

Conc. of STAS /plate/4.0 ml[a]	Av. no. XC plaques/plate	% XC plaque reduction[b]
1000	0	100
500	0	100
250	1	97
100	4	87
50	12	61
25	15	52
10	22	31
1	30	3
0.5	31	0
—	31	—[c]

[a] Culture plates were seeded with MEF cells as described in legend of Table I. Plates treated with STAS at the time of infection of culture with 50 PFU of MLV in 0.4 ml. Fluids were removed from cultures 3 days after virus infection. Cultures were re-fed with 4.0 ml of growth medium containing the appropriate concentration of STAS. Two plates of MEF cells were employed at each concentration of STAS.

[b] % plaque reduction = (av. no. of plaques on control plates − av. no. plaque on treated plates × 100)/av. no. plaques in control plates.

[c] MEF cell cultures infected with 50 PFU of MLV served as virus control.

TABLE III. MLV Replication in MEF Cell Cultures Treated with STAS at Various Times Before, Simultaneously with, and After MLV Infection.

Time of STAS treatment in relation to time at MLV infection (hr)[a]	Av. no. XC plaque/plate	% XC plaque reduction
−24	60	9
−2	66	0
0	0	100
+2	1	98
+24	6	91
+48	11	83
+72	29	56
—	66	—[c]

[a] Culture plates were seeded with MEF cells as described in legend of Table I. (−) is time of addition of 1000 μg of STAS/plates before MLV infection, (+) is time of addition of 1000 μg of STAS/plate after MLV infection. Cultures infected with 75 PFU at zero time period. Cultures were re-fed with fresh medium containing STAS 3 days after infection.

[b] See legend to Table II.

[c] Two plates of MEF cells infected with 75 PFU of MLV served as virus controls.

of the average MLV-induced plaque count seen in untreated MLV-infected MEF cultures (virus controls). In addition, the STAS induced inhibition of MLV plaque formation in MEF cell cultures appears to be directly proportional to the concentration of STAS employed.

Moloney leukemia virus replication was determined in MEF cell culture treated with STAS at various times before, simultaneously with, and after infection with MLV. Data presented in Table III show that MLV-infected cultures treated with STAS simultaneously with infection, or 2, 24, 48, or 72 hr after infection resulted in significant inhibition of MLV replication.

Chermann *et al.* reported that STAS prepared from the supernatant of a spontaneously transformed murine cell line, 8828, failed to inhibit M–MSV focus formation *in vitro* (3), while STAS preparations from beef and mouse spleen inhibited M–MSV focus formation *in vitro* (4). Thus, investigations were undertaken to determine the nature and distribution of the inhibitor present in STAS. Supernatant fluid from the virus-free murine cell line, JLS–V9, fetal calf serum (FCS), normal Balb/c mouse plasma, and Eagle's minimum essential medium supplemented with 10% FCS was carried through the procedure employed by Jasmin *et al.* (1) for the preparation of STAS. These preparations along with neutralized STA acid were tested for their ability to inhibit MLV replication *in vitro*. All preparations, including neutralized STA acid, produced significant inhibition of MLV replication *in vitro* (Table IV). Concomitantly, data in Table IV show that (i) STA was used in the preparation of all the inhibitor preps, (ii) neutralized silicotungstic acid alone resulted in a 92% reduction in the average plaque count seen in MLV control, and (iii) all preparations tested inhibited MLV replication at approximately the same

TABLE IV. Attempts to Determine the Active Ingredient in JLS–V5 STAS and Its Distribution.

Treatment of MEF cell cultures[a]	% XC plaque reduction[b]
JLS–V5 (STAS) + MLV	97
JLS–V9 (STAS) + MLV	100
Fetal calf serum (STAS) + MLV	100
Normal Balb/c mouse plasma (STAS) + MLV	100
MEM + 10% FCS (STAS) + MLV	100
Silicotungstic acid (neutralized) + MLV	92
MLV	—[c]

[a] Culture plate seeded with MEF as described in the legend of Table I. Cultures treated with 1000 μg of STAS preparation in 4.0 ml of growth medium at the time of infection with 50 PFU of MLV. Two culture plates of MEF were employed/sample.

[b] See legend to Table III.

[c] Two plates of MEF cells infected with 50 PFU of MLV served as virus controls.

level as JLS–V5 STAS. The data suggest that the inhibiting activity of STAS against MLV replication *in vitro* was probably due to the presence of residual or bound STA in the STAS preparation.

Data shown in Table V indicates that "mock" preparations containing saline + STA and processed by the procedure described by Jasmin *et al.* (1) with and without polyethylene glycol (PEG), significantly inhibited MLV replication in MEF cells. In addition, "mock" preparation of silicic acid and tungstic acid resulted in a 41% and 50% reduction of MLV plaque formation respectively. These data clearly show that silicotungstic acid can inhibit MLV replication *in vitro*.

In order to determine the presence of an inhibitory component other than STA in JLS–V5 supernatant, inhibitor was prepared from JLS–V5 supernatant by a procedure identical to the method described by Jasmin *et al.* (1) except that other protein-precipitating agents were substituted for STA.

Data shown on Table VI indicates that only preparations from JLS–V5 supernatant fluids employing $(NH_4)_2SO_4$ induced significant inhibition of MLV replication *in vitro* when compared to the MLV inhibitory capacity of STAS. This observation thus suggests the presence of an inhibitor in JLS–V5 supernatant which inhibits MLV replication *in vitro* that may not be associated with residual STA in the STAS preparation.

In an attempt to determine the ability of STAS to inactivate MLV directly, MLV was incubated with 800 μg of STAS at room temperature for 1 hr. The mixture was then centrifuged at 30,000*g* for 1 hr to pellet the virus. The virus pellet was resuspended to its original concentration and titrated by the XC assay in MEF cell culture along with nontreated nonpelleted virus, and nontreated pelletized virus. Treatment of virus with STAS, under the conditions stated, resulted in no significant reduction of titer of treated virus ($10^{6.0}$ PFU/ml) as compared with the titer of non-STAS-treated virus ($10^{6.4}$ PFU/ml). In contrast, Dienhardt has shown that Rous sarcoma virus (RSV) was inactivated when the virus was incubated with STAS for 3 hr at 37° (8).

Discussion. Chermann *et al.* demonstrated that JLS–V5 STAS markedly inhibited FLV leukemogenesis *in vivo* and M–MSV focus-forming activity *in vitro* (3). In confirmation of the latter observation, data reported in this communication show that STAS markedly inhibits MLV replication *in vitro,* while fail-

TABLE V. The Effect of "Mock" Inhibitors on MLV Replication *in Vitro*.

Treatment of MEF cell cultures[a]	% XC plaque reduction[b]
JLS–V5 (STAS) + MLV	100
Saline + PEG + silicotungstic acid + MLV	90
Saline — PEG + silicotungstic acid + MLV	100
Saline + silicic acid + MLV	41
Saline + tungstic acid + MLV	50
MLV	—[c]

[a] Plates treated with 1000 μg of the various preparations in 4.0 ml of growth medium at the time of infection with 75 PFU of MLV. Two culture plates of MEF cells were employed/sample.

[b] See legend in Table III.

[c] Two plates of MEF cells infected with 75 PFU of MLV served as virus controls.

TABLE VI. Inhibition of MLV Replication Induced by JLS–V5 Inhibitor Prepared Employing Various Protein Precipitating Agents.

Treatment of MEF cell cultures[a]	% XC plaque reduction[b]
JLS–V5 ($ZnSO_4$) + MLV	0
JLS–V5 (PCA) + MLV	6
JLS–V5 (TCA) + MLV	0
JLS–V5 (ETOH) + MLV	8
JLS–V5 (NH_4SO_4) + MLV	50
JLS–V5 (STAS) + MLV	100
MLV	—[c]

[a] Cultures treated with 1000 μg of inhibitor preparations in 4.0 ml of growth medium at the time of infection with 75 PFU of MLV. Two culture plates of MEF cells was employed/sample.

[b] See legend to Table III.

[c] Two plates of MEF cells infected with 75 PFU of MLV served as virus controls.

ing to significantly inactivate MLV directly. However, in contrast to data reported by Jasmin *et al.* (1), and Chermann *et al.* (3), data presented in this report suggest that the viral inhibitory component of STAS is STA. The implication of STA as the viral inhibitory component of STAS was further strengthened by data which clearly demonstrated the ability of STA to inhibit MLV replication *in vitro,* and the inability of STA to be completely removed from STAS preparation by prolonged dialysis against distilled water. The possible existence of a biological inhibitor in supernatant fluids from JLS–V5 cells was suggested by results which showed that an inhibitor prepared employing $(NH_4)SO_4$ induced significant inhibition of MLV replication *in vitro.*

Recently, Raynaud *et al.* have reported the ability of STA to inhibit focus-forming activity of M–MSV *in vitro* (9). Thus, this report and the report of Raynaud *et al.* (9) clearly show that STA inhibits both murine leukemia and sarcoma viruses *in vitro.*

A clear confirmation of the presence in JLS–V5 supernatant fluids of a new biological inhibitor which markedly inhibits murine leukemia and sarcoma virus replication *in vitro* was not obtained in this laboratory. In addition, treatment of mice with the various STAS preparations failed to protect mice from the leukemogenic activity of FLV or a Rauscher ascites tumor line (10).

Summary. An inhibitor (STAS) prepared from the supernatant of JLS–V5 cells blocked the replication of MLV *in vitro* when added to MEF cultures as late as 48 hr after but not prior to infection with MLV. Potent antiviral activity against MLV was also observed with inhibitor prepared from JLS–V9 tissue culture supernatant fluid, fetal calf serum (FCS), normal Balb/c mouse plasma, MEM + 10% FCS, saline, or silicotungstic acid (STA). Inhibitor prepared from JLS–V5 supernatant fluid, but substituting $ZnSO_4$, PCA, TCA, ethanol, or ammonium sulfate precipitants instead of STA, showed little or no activity against MLV *in vitro.* No viracidal activity was obtained when MLV was exposed directly to JLS–V5 STAS. These studies suggest that the active ingredient in JLS–V5 STAS may be silicotungstic acid.

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