forty-seven of 905 eggs from 41 of 92 chicken flocks in 12 states contained the antibody. Although there was considerable geographic variation consistent with sampling problems, it is apparent that this is a common virus infection of North American chickens. Results of the cross-immunofluorescence test confirmed that REV is not a member of the well-characterized sarcoma-leukosis group of avian viruses.

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Effect of Statolon on Acute and Persistent Murine Leukemia and Sarcoma Virus Infections (33517)

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Statolon, a product of a mould, *Penicillium stoloniferum*, has been shown to exert its antiviral action by stimulating the *in vitro* and *in vivo* production of interferon (1,2). Statolon, now known as double-stranded phage RNA (3, 4), was recently reported to inhibit Friend leukemia virus infection in mice (5, 6). Statolon inhibits Friend leukemia virus when administered as early as 6 days before, and as late as 9 days after, virus inoculation. The majority of statolon-treated mice are completely protected against Friend virus leukemia and are resistant to rechallenge with the virus (6). However, no studies of the effect of statolon on murine leukemia and sarcoma virus infection of tissue cultures have been reported.

In the present communication, we describe the effect of Statolon on acute and persistent infection of tissue cultures with murine leukemia and sarcoma viruses. Statolon inhibits not only the growth of murine leukemia virus, but also the *in vitro* cellular transformation induced by murine sarcoma viruses.

Materials and Methods. Viruses. The tissue culture grown murine leukemia and the Moloney strain of murine sarcoma virus (M-MSV) used were furnished by Dr. J. W. Hartley. These had been serially passed by Hartley in Swiss NIH mouse embryo tissue culture (NIH-METC) (7) and were passed by us several times further in the same type of tissue culture prior to the studies described below. MSV pseudotypes (8) were made into virus stock as Moloney procedure concentrates (9) of mouse tumors.

Vesicular stomatitis virus (VSV) was grown in mouse L cell monolayer cultures. Reovirus type 3 (Dearing) was grown in a line of African green monkey kidney cells (Vero) (10).

Cell cultures. Primary cultures of NIH-METC were prepared as previously described (7). Growth medium was 10% fetal bovine serum in Eagle's minimum essential medium (10% FBS-EMEM) with 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin/ml. This medium was also used for maintenance of cultures used for production of leukemia virus, and for complement-fixing (CF) antigen pools.

The Vero cells were subcultured and maintained in medium 199 supplemented with 5% fetal bovine serum (10). A continuous line of Rauscher leukemia virus-infected rat embryo cells (11) was also used in this study.

Antigen and virus pools. To prepare CF antigen grown in NIH-METC, 14- to 21-day-

old infected cultures were harvested by scraping the cells from the surface of the cultures and making 10% cell pack preparations. Before testing for CF activity, cell suspensions were frozen and thawed once, and were sonicated in a Branson sonifier (model S-75) for 3 sec at 3 amp. Culture media 7 or more days old) from infected cultures were removed without disturbing the cell sheet and used as a source of seed virus. The infectious virus was assayed in the CF test (7).

Virus assay. Virus titrations for murine leukemia viruses were done in disposable 60-mm plastic petri dishes of secondary NIH-METC, employing 2 plates/10-fold dilution by CF antigen induction test (7). The sarcoma virus was also assayed in secondary NIH-METC plate cultures. Altered cell foci (12) were counted with an inverted Zeiss microscope 6–8 days after sarcoma virus inoculation. Reovirus type 3 was assayed in Vero cells by the plaque method previously described (10).

Assay of interferon. Interferon was assayed in L cell monolayers by the vesicular stomatitis virus yield reduction method (13).

Complement-fixation (CF). Complement fixation tests were carried out in the microtiter technique described for tumor antigen studies (14). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement. Rat antisera used in the CF test were obtained from Fisher rats carrying transplanted sarcomas induced by the Moloney isolate of mouse sarcoma virus (M-MSV) (15). Antigen titrations were carried out by using 1:20 dilutions of anti-MSV rat serum containing 4 units of antibody as determined by titration against murine leukemia virus antigen prepared in NIH-METC. The serum pool used did not react when tested at a 1:10 dilution against a variety of normal mouse and rat tissue extracts and tissue culture cell antigens.

Statolon preparations. The statolon used in this experiment was supplied by the kindness of Dr. W. J. Kleinschmidt, The Lilly Research Laboratory, Indianapolis, Indiana. Stock solution was prepared as 1 mg/ml of statolon diluted in 2% FBS-EMEM with 100 units of penicillin, 100 μg of streptomycin, 50 units of polymyxin B sulfate (Aerosporin) and 25 units of Mycostatin /ml. For the experiments, the statolon was further diluted into different concentrations, using growth medium as diluent.

Mouse interferon used in this experiment was kindly supplied by Dr. S. Baron.

Results. Effect of statolon on vesicular stomatitis virus and reovirus. The inhibitory effect of statolon and mouse interferon on VSV and reovirus was tested. With 10^5 PFU of VSV/ bottle (approximate multiplicity of infection = 0.5), there was delay in the development of CPE of VSV in NIH-METC pretreated with statolon (100 μg/ml) and mouse interferon (35 units) for 24 hr as compared with untreated NIH-METC. There was a 2-log lower yield of VSV in pretreated cultures. With 10^6 PFU of reovirus/bittle (approximate multiplicity of infection = 5), there was also definite delay in the development of CPE of reovirus in pretreated NIH-METC, as compared with untreated cultures. There was a 2-log lower yield of reovirus in statolon-treated cultures and a 1-log reduction of yield in cultures treated with mouse interferon, as compared with the untreated.

Effect of Statolon and mouse interferon on Friend leukemia virus and mouse sarcoma virus. Using the same concentrations as in the above experiment, the effect of statolon on Friend leukemia virus and mouse sarcoma virus was examined. Three sets of two 10-oz bottles of NIH-METC were treated at the same time with statolon, mouse interferon, or the growth medium. After 24-hr incubation, the bottles were washed twice with HBSS, inoculated with 10^5 infectious units of virus per bottle, and absorbed for 2 hr at 37°. The cultures were then washed 5 times before the growth medium was added and incubated for 21 days at 37° in a humified air atmosphere containing 5% CO_2. Fluids were removed twice a week, when the medium was changed, and stored at —70°. One of each set was harvested 14 days after inoculation, the other after 21 days. The fluids were assayed for infectivity, and the cells were packed for CF

TABLE I. Cell Resistance to Friend Leukemia Virus in Mouse Embryo Cells Treated with Statolon and Mouse Interferon.[a]

	Days after inoculation					
	7		14		21	
Treatment	CF titer	Infectivity[b] titer	CF titer	Infectivity titer	CF titer	Infectivity titer
Untreated	—[c]	3.4	4	3.4	4	3.4
Statolon treated	—	<1.4	<1	<1.4	<1	<1.4
Mouse interferon treated	—	3.4	(4)	2.4	4	3.4

[a] Cultures were treated for 24 hr with statolon (100 μg/ml) or mouse interferon (35 units/ml) before inoculation of the virus. Two logs of VSV inhibition was achieved by these materials.
[b] CF induction titer per ml.
[c] — = not done.

antigens by the method described above.

Table I shows the effect of statolon and mouse interferon on Friend leukemia virus in NIH-METC. Complement-fixing antigen titer was 1:4 after 14 days' incubation in untreated cultures and in mouse interferon treated cultures, and remained the same after 21 days' incubation. However, no CF antigen was detected in the statolon pretreated cultures after 14 and 21 days' incubation. In the untreated and mouse interferon pretreated cultures, 3.4 logs of virus were present, but in the statolon-treated ones, no Friend leukemia virus was detected at the 1:10 dilution tested (Table I).

The inhibitory effect of statolon on mouse sarcoma virus (M-MSV) was also observed (Table II). In the cultures of untreated cells, as well as in those treated with mouse interferon, there was a CF antigen titer of 1:8, whereas there was no CF antigen in the statolon-treated cultures. In the statolon-pretreated cultures, the yield of M-MSV was also reduced by at least 2 logs. The lack of inhibitory effect of mouse interferon on Friend leukemia and mouse sarcoma virus may have been due to the small doses used in these experiments and probably also due to 21-day virus growth period after removal of interferon.

Effect of statolon on cellular transformation induced by murine sarcoma viruses. Figure 1 shows the focus forming and CF titers of M-MSV (ML) as a function of statolon concentration used to pretreat the cells for 24 hr. A significant inhibition of the viral trans-

TABLE II. Cell Resistance to Moloney Sarcoma Virus in Mouse Embryo Cells Treated with Statolon and Mouse Interferon.[a]

	Days after inoculation					
	7		14		21	
Treatment	CF titer	Infectivity[b] titer	CF titer	Infectivity titer	CF titer	Infectivity titer
Untreated	—[c]	3.4	8	3.4	8	3.4
Statolon treated	—	<1.4	<1	<1.4	<1	1.4
Mouse interferon treated	—	3.4	8	3.4	8	3.4

[a] Cultures were treated for 24 hr with statolon (100 μg/ml) or mouse interferon (35 units/ml) before inoculation of the virus. Two logs of VSV inhibition was achieved by these materials.
[b] CF induction titer per ml.
[c] — = not done.

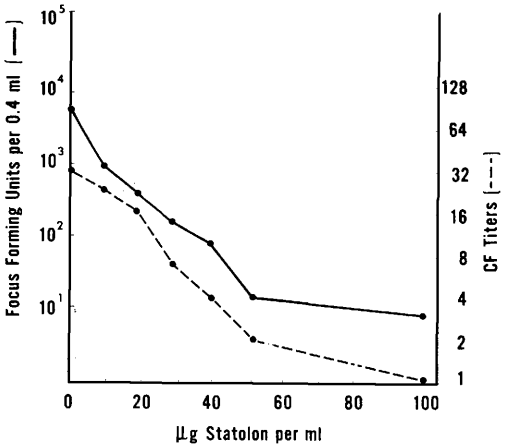

FIG. 1. Focus-forming and CF titers of M-MSV (ML) as a function of the statolon concentration used to pretreat mouse embryo tissue culture. (These data were obtained by averaging the titers of two experiments.)

forming activity was caused by statolon. This effect was maximal with about 100 µg of statolon/ml. Higher concentrations of statolon were toxic for the mouse embryo cells.

Table III shows the effect of statolon on various pseudotypes of murine sarcoma viruses. Inhibition of focus formation was observed with various MSV pseudotypes tested.

Table IV lists the inhibitions of focus formation and CF antigen of M-MSV(ML) when statolon was given at various times. Four tests were conducted in which statolon was added (i) 24 hr before the virus, (ii) at the same time as the virus, (iii) 24 hr after the virus, and (iv) 48 hr after the virus. In each case, statolon was added for only 24 hr, after which the cultures were washed three times and the media replaced. Statolon was effective for 24 hr before and after infection. The high values of inhibition were obtained when statolon was provided 24 hr before infection. When statolon was added 48 hr after the virus, no significant inhibition was obtained as shown in titers of focus formation and CF antigen of M-MSV(ML). It was shown earlier that optimal protection against infectious viruses in mice was obtained when statolon was administered intraperitoneally 24 hr prior to virus challenge (16).

These tests gave some indications of tissue toxicity with higher concentrations of statolon, particularly in those of 100 µg/ml or greater. However, the toxicity may be due to one of the many extraneous materials in the impure preparation of statolon. In these tests, we also noted that autoclaved (15 lb for 15 min) statolon, even in fairly strong concentration (50 µg/ml) had no inhibitory action against M-MSV(ML). This heat lability has been shown previously in both mouse and tissue culture tests (1, 2, 17).

A virucidal effect was ruled out by adding statolon to a viral suspension. Data show that statolon, at the concentrations used in these studies, was not virucidal to M-MSV(ML) in suspension. These experiments clearly demonstrate that statolon does not act directly on the virus, but exerts its action on the host cell, as reported by others (18).

Effect of statolon on Rauscher leukemia virus as a persistent infection of rat embryo cell cultures. Experiments were designed to

TABLE III. Effect of Statolon on Focus Formation by Mouse Sarcoma Viruses.

Mouse sarcoma virus pseudotypes[b]	Titer without statolon (FFU/0.4 ml)	Titer with statolon[a] ($\times 10^1$; FFU/0.4 ml)	Log 10 inhibition by statolon
MSV (ML)	3.8×10^3	2.0	2.3
MSV (RL)	2.8×10^3	<1.0	>2.4
MSV (GL)	8.0×10^1	<1.0	>0.9
MSV (FL)	2.2×10^3	<1.0	>2.3

[a] Statolon was added to the nutrient medium at a final concentration of 100 µg/ml and left in contact with the cells for 24 hr. Immediately before infection the statolon medium was removed, the cells were rinsed twice with nutrient medium and supplied with fresh nutrient medium.

[b] Virus types and abbreviations are the same as in a previous communication (17).

TABLE IV. Exposure of Cell Cultures to Statolon[a] at Various Times: Inhibition of Focus Formation and CF Antigen of MSV(ML).

Titer		Statolon exposure time relative to infection			
		Added 24 hr before virus	Added to cells at same time as virus	Added 24 hr after virus	Added 48 hr after virus
Infectivity titer[b] (FFU/0.4 ml)	of control	1.8×10^3	2.4×10^3	1.9×10^3	1.9×10^3
	of statolon-treated cells	$<1.0 \times 10^1$	3.0×10^2	3.5×10^2	5.0×10^2
CF titer[c]	of control	1:16	1:16	1:16	1:8
	of statolon-treated cells	1:1	1:2	1:2	1:8

[a] Statolon (50 μg/ml) treated for 24 hr.
[b] Focus-forming unit was determined 6 days after inoculation.
[c] CF antigen titer 14 days after inoculation.

examine the effect of a single 24-hr treatment with statolon on Rauscher leukemia virus infected rat embryo cell cultures.

Twenty plate cultures were made from twentieth passage of Rauscher leukemia virus infected rat embryo cultures, and incubated for 8 days at 37°. Then each 4 plates were treated with different concentrations of statolon (see Table V) for 24 hr. The statolon was removed, the cells were rinsed twice with nutrient medium and supplied with fresh medium. The cultures were incubated at 37° in a CO_2 incubator and were harvested, 2 plates after 6 days of incubation and 2 plates after 14 days. A single treatment with statolon (100 μg/ml) decreased the CF titer more than four times in a 2-week interval (Table V).

TABLE V. Effect of Single Treatment with Statolon on Persistent Infection of Rauscher Leukemia Virus in Rat Embryo Cells.[a]

Statolon (μg/ml)	Harvested days after treatment	CF antigen titers vs MSV rat serum
0	6	128
	14	>64
10	6	64
	14	>64
25	6	(64)
	14	64
50	6	64
	14	>64
100	6	32
	14	32

[a] Four plates each of 8-day-old cultures of Rauscher leukemia virus infected rat embryo cells (twentieth passage) were treated for 24 hr with different concentrations of statolon. Then the statolon medium was removed, the cells were rinsed twice with nutrient medium, and supplied with fresh nutrient medium. The CF titer of 8-day-old cultures of Rauscher leukemia virus-infected rat embryo cells was 1:32.

Further experiments were designed to examine the effect of prolonged treatment with statolon on persistent Rauscher leukemia virus infection in rat embryo cells. Twenty-four-hr-old plate cultures of Rauscher leukemia virus-infected rat embryo cultures were fed with growth medium containing different concentrations of statolon and incubated at 37°. Two plates from each group were harvested for CF antigen. The remaining plates were replenished again with the growth medium containing statolon and further incubated. Eightfold inhibition of CF titers was observed in the cultures containing 50 μg/ml of statolon (Table VI). However, there were definite toxic effects in the cultures containing more than 50 μg/ml of statolon after 7 days of incubation. We therefore continued our examinations at lower concentrations. Seven-day-old cultures of the rat embryo cells (twenty-fifth passage) were fed with medium containing 12.5 and 25 μg/ml of statolon. After various periods of incubation, the plates in each group were harvested and

TABLE VI. Effect of Prolonged Treatment with Statolon on Persistent Infection with Rauscher Leukemia Virus.[f]

Rauscher leukemia virus-infected culture	Statolon (μg/ml)	Harvested days after addition of statolon	CF[c] antigen titers	Infectivity[d] titer
23rd passage[a]	0	4	16	—[e]
		7	32	$>10^{3.7}$
	25	4	4	—
		7	4	$10^{2.7}$
	50	4	<4	—
		7	4	$10^{1.7}$
	100	4	<4	—
		7	<4	$10^{0.7}$
25th passage[b]	0	5	16	—
		8	32	—
		11	(64)	$10^{4.7}$
	12.5	5	8	—
		8	(16)	—
		11	16	$10^{2.7}$
	25	5	8	—
		8	8	—
		11	8	$10^{1.7}$

[a] One-day-old cultures.

[b] Seven-day-old cultures, CF 1:8; infectivity titer, $10^{1.7}$.

[c] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement (in parentheses indicates 2+).

[d] CF induction titer ($\log_{10}$ TCD50/ml).

[e] — = not done.

[f] Rauscher leukemia virus-infected rat embryo cultures in nutrient medium were replenished with the statolon medium and incubated at 37° in a CO_2 incubator.

tested for CF antigen. Table IV shows that complete inhibition was achieved with the medium containing 25 μg/ml of statolon. No increase of infectivity or of CF titers of Rauscher leukemia virus in rat embryo cells were observed during prolonged treatment with statolon.

Discussion and Summary. Statolon, known to induce interferon, inhibited not only the growth of murine leukemia viruses (Friend and Rauscher) in NIH-METC, but also the *in vitro* cellular transformation induced by murine sarcoma viruses.

The results obtained on the inhibitory effect of statolon against murine leukemia and sarcoma viruses in tissue culture were in agreement with *in vivo* results obtained previously by others (5, 6, 16, 18). Best results of statolon against murine sarcoma viruses were obtained by single pretreatment of the cells with statolon (50 μg/ml) for 24 hr, followed by virus inoculation; but inhibition was also observed when the drug was added at the same time as the virus, or 24 hr later, in a single treatment. As reported herein, a were obtained by single pretreatment of the single treatment of statolon protected mouse embryo cells against Friend leukemia virus infection for at least 3 weeks. It has been shown that optimal protection against MM and Semliki Forest viruses in mice was obtained when statolon was administered 24 hr prior to virus challenge (16) and one injection of statolon afforded significant protection in the mouse for at least a month (18).

The results of the experiment on persistent infection indicated that prolonged treatment with statolon can protect rat embryo cells

against persistent infection with Rauscher leukemia virus (Table VI). Gresser has reported the inhibition of Friend virus induced splenomegaly by repeated intraperitoneal administration of an interferon preparation (personal communication).

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