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Effect of Silica on Virus Infections in Mice and Mouse Tissue Culture

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Silica injections of mice have been reported to kill macrophages, thus allowing herpes simplex virus (HSV) to spread rapidly and leading to an increased severity of HSV infection. Thus, silica presumably could be used to eliminate lactic dehydrogenase virus (LDV) (a model for slow viruses), which is known to multiply exclusively in macrophages. Contrary to expectation, it was found that the LDV titers were increased in silica-injected mice as compared to the titers in control mice. Counts of peritoneal cells at different periods after silica injection showed that silica-induced macrophage damage in vivo resulted in proliferation and migration of macrophages, thus providing additional target cells for LDV replication and leading to high LDV titers. In vitro, silica ingestion also damaged the macrophages, but since no replacement of cells could occur by infiltration, decreased LDV titers were found. Similar findings were obtained with HSV. It is suggested that all persistent viruses multiplying in macrophages will show a similar recrudescence under comparable conditions.

Previously, evidence has been presented that lactic dehydrogenase virus (LDV) replicates mainly or perhaps exclusively in mouse macrophages (4, 6, 12, 13). In a number of studies it was reported that the administration of silica impaired macrophage function or killed macrophages (2; for further references see 7, 8, 10, 14, and 17). Furthermore, Zisman et al. (19) reported that as a result of silica impairment of macrophages the HFEM strain of herpes simplex virus (HSV), which normally is retained by macrophages, was allowed to spread to liver parenchyma, leading to early death of the mice. Silica was therefore used to define the role of macrophages during LDV and HSV infection in vivo and in vitro.

MATERIALS AND METHODS

Animals. Randomly bred Swiss albino mice (G.P., female), 20 to 30 g, were supplied by the National Institutes of Health animal production center.

Viruses. The strain of LDV used was isolated from a naturally infected Ehrlich ascites carcinoma and carried in CDF1 mice, and its titer was determined by a modification of the pooled plasma method as described before (5). Stocks of type 1 HSV (strain VR4) were grown in primary rabbit kidney cell monolayers, and the virus titer was measured by determination of its cytopathic effect on BHK-21 cells, grown in Eagle minimal essential medium with 2% fetal bovine serum in microtiter plates.

Media. Three media were used: macrophage collecting medium (MCM), medium 199 containing 100 U of penicillin and streptomycin and 1 USP unit of heparin per ml; macrophage wash medium (MWM), MCM without heparin; and macrophage growth medium (MGM), MWM plus 20% fetal bovine serum, not inactivated. Fetal bovine serum was obtained from Industrial Biological Laboratories, Rockville, Md. Liquid paraffin, drakeol 6-Vr (pharmaceutical grade), was obtained from the Pennsylvania Refining Co., Butler, Pa., and was sterilized before use. Silica was kindly supplied by A. C. Allison, representing an international standard sample of quartz (Dorentrup no. 12, particle size up to 5 μm) which, as suggested, was heat sterilized and then dispersed by ultrasonic treatment in saline for 1 min.

Macrophages. Macrophages were obtained from the peritoneal cavity of mice, untreated or preinjected with silica, drakeol, and/or viruses, and were cultured in test tubes as described previously (3). Cell counts were made (i) of the original peritoneal wash (four to five mice per set, 4 ml of MCM per mouse) and (ii) of the resuspended cells, obtained after centrifugation of the wash at 500 $\times$ g for 10 min in a refrigerated international centrifuge and resuspended in MWM to the original volume of the peritoneal wash. Counts were also made of the cells which had adhered to cover slips (25 by 25 mm) which had been placed in Falcon plastic petri dishes (30 by 10 mm). One milliliter of resuspended cell suspension was added to each dish. After 2 to 4 h the nonadherent cells were removed by washing with MWM and the remaining cells were cultured for another 24 h in MGM. Then the dishes with cover slips were washed again, dried at 37 C for 12 h, and stained with Giemsa stain, and the number of cells per microscopic field were counted under $\times$300 magnification, thus eliminating nonadhering and temporarily adhering B lymphocytes. Counts, including viability counts with 0.05% trypan blue, were

further made of the subpopulation of macrophages, prepared as described above but in petri dishes without cover slips. The subpopulation of adherent cells which had specific characteristics of macrophages were identified by their surface receptors for the Fc fragment of 7S immunoglobulin, bound to sheep erythrocytes (SRBC) by a modification of the method of Abramson et al. (1). After exposure of the settled cells to the SRBC for 30 min at 37 C in veronal buffer (pH 7.4), the cells were washed with phosphate-buffered saline, fixed with 1% phosphate-buffered glutaraldehyde (pH 7.2), stained with Giemsa, and counted as described above.

RESULTS

In vivo effect of silica injections. Silica was injected either intraperitoneally (i.p.), 20 or 50 mg/mouse, or intravenously (i.v.), 3 or 15 mg/mouse. Twenty-four hours later, groups of mice were infected i.p. with increasing doses of HSV or with 10^7 mean infective doses of LDV per mouse.

Table 1 presents the results of the LDV experiments. It can be seen that the i.p.-administered silica increased the LDV titer by about 10-fold over the controls, 12 h after infection, followed by a leveling off to control values. The i.v. administration of silica led to a similar increase starting 1 day after infection and lasting 2 to 5 days.

Figure 1 presents the results of a representative HSV experiment with the VR_4 strain of HSV, to determine whether the VR_4 strain would yield the same results as the HFEM strain used previously (19). It can be seen that 10^3 mean tissue culture infective doses ($TCID_{50}$) of the HSV killed 60% of the mice, whereas 10^2 and 10^1 $TCID_{50}$ were mainly nonlethal. With the silica given i.p., both 10^2 and 10^1 $TCID_{50}$ per mouse were 100% lethal, whereas with the silica given i.v. a dose of 10^2 $TCID_{50}$ of HSV was 50% lethal. Thus, silica preinjection increased the lethality of HSV about 100-fold. The results with HSV seem to corroborate the interpretation that the silica, by disabling the macrophages, allowed the HSV to reach the primary target cells, leading to mortality (19). On the other hand, the results with LDV (Table 1) indicated that the virus growth was enhanced in silica-treated animals which reportedly contained fewer macrophage host cells for LDV. This raised the possibility that silica did not kill macrophages.

Therefore, the next experiments were designed to answer the question whether the silica would kill macrophages in vitro.

In vitro effect of silica on peritoneal macrophages and on its effect on LDV and HSV multiplication in these cell cultures. Cultures of peritoneal macrophages were prepared in test tubes as described. After collection (24 h), the growth medium was replaced by medium containing 500, 100, 50, 10, and 0 μg of silica per tube (1 ml). After 24 h, the cultures were infected with LDV or HSV.

The effect of silica on macrophage survival was measured by estimating the percentage of rounded cells in a given area (one microscopic field at a given magnification) and the percentage of the same area covered by the cells (Table 2). The effect of the silica on the virus multiplication was determined by the titer of the culture supernatants at various time intervals after inoculation (Table 3). Increasing amounts of silica caused a progressive rounding of the cells concomitant with a progressive decrease of glass-adhering cells (Table 2). The damage by silica and by HSV to the cells was cumulative (Table 2). (Since LDV does not cause rounding of cells and loss of adherence, the LDV-infected, silica-treated series was used as control for the silica effect.) Paralleling the results of Table 2, the virus titers progressively decreased when

TABLE 1. *Effect of silica on LDV titers (log_{10} mean infective doses per ml ± standard deviation) in mice*

Expt	12 h	Day 1	Day 2	Day 5	Day 6	Day 9	Day 14
1[a]							
Control	8.5 ± 0.20	9.3 ± 0.20	8.7 ± 0.20		7.9 ± 0.32		7.5 ± 0.20
Silica i.p.[b]	9.5 ± 0.20	9.5 ± 0.28	8.1 ± 0.24		7.3 ± 0.20		6.9 ± 0.24
Silica i.v.[c]	9.5 ± 0.20	10.7 ± 0.20	9.7 ± 0.20		7.7 ± 0.32		6.7 ± 0.20
2							
Control	8.9 ± 0.32	9.9 ± 0.28	8.9 ± 0.28	7.5 ± 0.37		7.1 ± 0.32	
Silica i.p.[d]	9.7 ± 0.32	9.7 ± 0.35	9.7 ± 0.20	7.9 ± 0.24		8.5 ± 0.28	
Silica i.v.[e]	8.9 ± 0.24	10.9 ± 0.24	10.1 ± 0.24	9.3 ± 0.20		7.3 ± 0.20	

[a] Mice infected with 10^7 mean infective doses of LDV per mouse (i.p.) 24 h after injection of silica.
[b] i.p. injection, 20 mg of silica per mouse.
[c] i.v. injection, 3 mg of silica per mouse.
[d] i.p. injection, 50 mg of silica per mouse.
[e] i.v. injection, 15 mg of silica per mouse.

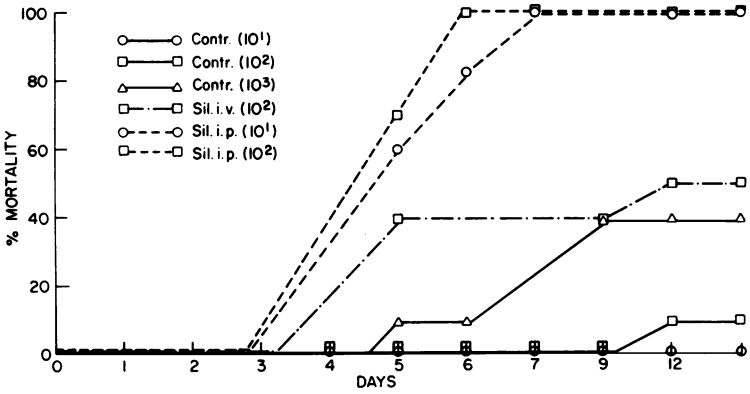

FIG. 1. *Effect of silica, given i.p. or i.v., on the percent mortality of mice infected with HSV. On day* t_{-1}, *groups of 12 mice were injected with 50 mg of silica i.p., or 15 mg of silica i.v. On day* t_0, *mice were infected with HSV,* 10^3, 10^2, *or* 10^1 $TCID_{50}$ *per mouse (numbers in brackets on figure).*

TABLE 2. *Effect of silica on mouse macrophages survival in vitro at various times (days) after injection of silica*

Virus	Silica (μg/ml)	Day 1	Day 2	Day 3	Day 5	Day 7	Day 12
LDV set	0	10/99[a]	10/90	20/90			20/90
	10	15/99	10/80	20/80			20/80
	50	40/70	50/80	40/70			50/50
	100	60/95	50/50	50/50			60/30
	500	40/95	20/80	50/50			60/50
HSV set	0	10/90	10/95	20/80	30/70		70/20
	10	10/80	15/90	20/70	30/70		90/10
	50	60/40	60/40	40/30	50/30		90/20
	100	50/50	70/40	30/80	40/50		95/20
	500	90/70	80/60	80/60	80/60		97/20

[a] Index, Percentage of cells rounded/percentage of area covered by cells, per microscopy field at a given magnification.

the amount of silica was increased (Table 3). The experiments on the effect of silica on virus titers were repeated by using primary mouse embryo tissue culture, which did not phagocytose silica. No deleterious effect of the silica on HSV multiplication was observed.

Thus, the question arose as to why the virus titers increased after silica treatment in vivo and decreased after silica addition in vitro. To determine whether this increase might be due to some unknown stimulating effect of silica on macrophages when administered in vivo, experiments were performed on the growth of virus in macrophages in vitro, obtained from animals which had been injected in vivo with silica. Mice were injected i.p. with 50 mg of silica per mouse. After 2 days, the peritoneal macrophages were collected and cultured. After 1 day in culture, when the macrophages were morphologically intact, the cells were infected with HSV and the virus titers were determined in the culture supernatant 4 days after infection. After 4 days the control samples contained virus, but the macrophages from the silica-treated mice were rounded and contained no measurable virus. These results, supported by microscopic observations and viability counts which decreased in time, showed that peritoneal macrophages containing silica went on to die when explanted. However, it remained possible that in vivo the irritation caused by silica eventually stimulated the accumulation of enough new peritoneal macrophages to support increased virus growth.

Effect of silica and drakeol on peritoneal macrophage populations. To test the above-mentioned possibility, mice were injected i.p. with silica and a day later with LDV or HSV. Peritoneal washes were obtained 1 and 3 days after infection. Cell counts were made of the total number of suspended cells and of those cells which after settling for about 2 h still adhered to the glass after washing. For comparison data were obtained from mice receiving only

saline and from mice receiving drakeol, a stimulant of peritoneal exudates, i.p. Also, portions of the collected cells were tested for LDV and HSV titers. The data obtained from a representative experiment are given below.

Presented are the original cell count of the peritoneal wash of four mice (each washed with 4 ml of MCM) and of cells resuspended to the original volume in MWM after centrifugation (Table 4). Further, the total cell counts and those of the large cells (macrophages) were compared with the counts of the cells which adhered after 24 h of culture. After the cells had settled, they were washed and exposed to SRBC coated with rabbit anti-SRBC immunoglobulin 7S, and the macrophages among these adherent cells recognizable by rosette formation with SRBC were counted (Table 5).

After 1 or 3 days, the macrophage count in drakeol-treated mice was about two to three times the control count, and the count in silica-treated mice was four to six times the control count (Table 4). The counts of the original peritoneal wash and of the resuspended

TABLE 3. *Effect of silica added in vitro, 18 h before infection, on LDV and HSV titers in mouse macrophage cultures*

Silica added (μg/ml)[a]	Samples collected after infection						
	5th wash	Day 1	Day 2	Day 3	Day 4	Day 5	Day 12
LDV titer							
0	1.9 ± 0.24	7.3 ± 0.37				7.7 ± 0.2	4.5 ± 0.28
10		7.1 ± 0.24				7.1 ± 0.32	4.1 ± 0.32
50		5.5 ± 0.28				5.9 ± 0.28	5.7 ± 0.32
100		5.1 ± 0.24				2.3 ± 0.2	<0.5
500		3.9 ± 0.24				<0.5	<0.5
HSV titer							
0			2.75	2.00	>3	>3.50	
10			2.00	3.00	3.25	>3.50	
50			1.75	1.75	2	3.0	
100			3.50	0.75	<0	<0	
500			2.00	1.75	<0	<0	

[a] LDV titer are expressed in $\log_{10}$ mean infective doses per milliliter ± standard deviation in supernatants. HSV titers are expressed in $\log_{10}$ $TDIC_{50}$ per milliliter.

TABLE 4. *Effect of drakeol and silica on the total number of cells in peritoneal washes*[a]

Virus	Day 1		Day 3	
	Original count[b]	Resuspended count[c]	Original count	Resuspended count
None				
Control	3.55 ± 0.1	2.6 ± 0.3	3.55 ± 0.1	2.9
Drakeol	7.6 ± 1.0	7.8 ± 2.7	8.1 ± 0.5	10.4
Silica	17.8 ± 0.7	20.2 ± 1.1	17.2 ± 0.7	21.2
HSV				
Control	6.0 ± 0.05	5.9	3.9 ± 0.1	3.8
Drakeol	12.1 ± 0.15	12.2	11.4 ± 1.1	10.3
Silica	23.0 ± 0.5	22.1	14.6 ± 3.0	11.6
LDV				
Control	2.9 ± 0.35	2.5	4.2 ± 0.3	3.9
Drakeol	5.2 ± 1.0	5.3	6.0 ± 0.25	5.7
Silica	12.4 ± 0.3	10.9	13.1 ± 0.5	13.6

[a] Groups of 18 mice were injected i.p., either with saline, with 50 mg of silica per mouse, or with 1 ml of sterilized drakeol per mouse. Later (24 h), subgroups were injected with 10^5 $TCID_{50}$ of HSV per mouse or with 10^7 mean infective doses of LDV per mouse. 1 and 3 days after infection the peritoneal cells were collected.

[b] Average cell counts in millions per milliliter, ± standard error. Counts made with Levy counter.

[c] Resuspended after centrifugation of the original suspension to determine that there is no cell loss during manipulation.

cells, as well as the counts of cells recognizable as macrophages by their rosette formation with SRBC, were increased in the preparations obtained from the drakeol and silica-injected mice, as compared to those obtained from the control mice (Table 5).

As stated above, the cultures were tested for their LDV and HSV titers. The virus titers were determined in portions of the blood plasma, the sonically treated peritoneal wash, the supernatants obtained after centrifugation of the sonically treated material, and the sonically treated resuspended cells (Table 6). Contrary to the results obtained with the macrophages treated with silica in vitro, the virus titers in the fractions obtained from the silica- and drakenol-injected LDV- and HSV-infected mice were statistically significantly higher than those of the control mice. The titers of the peritoneal washes of the silica-treated mice were the highest, and those of drakeol-treated mice showed intermediary values. This correlates with the relative increase in macrophages, after either silica or drakeol injection (Tables 4 and 5).

The mortality curves of a second group of mice treated similarly as those used to obtain the cells for counts, i.e., infected 24 h after silica or drakeol injection with HSV (10^5 $TCID_{50}$ per mouse, i.p.), are presented in Fig. 2. These results correlate with the data on the silica-induced increased virus titers (Table 6) in that the increase in peritoneal cells led to an increase in HSV titers, which, in its turn, correlated with an increase in mortality.

In vivo duration of the rise in cell counts in peritoneal washes after silica injection. To determine how long the effect of the injection of silica on the peritoneal macrophage population lasted and thus might effect the viremia, the number of peritoneal cells was determined at increasing time intervals after i.p. injection of 50 mg of silica per mouse (Fig. 3). A significant rise in cell numbers over the control occurred 6 h after injection and, after reaching a maximum after 1 day, remained elevated for about 5 days before the counts returned to normal. Thus, the experiments reported on in this paper, which were performed 1 to 3 days after injection of silica, fell within the period of substantial rise in cell counts.

TABLE 5. *Effect of drakeol and silica on peritoneal cell populations*[a]

Determinant	Original wash		Resuspended cells		Settled cells	
	Total count[b]	Large cells[b]	Total count[b]	Large cells[b]	Total count[c]	With rosettes[c]
Control	1.3 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	0.9 ± 0.2	49.3 ± 2.0	42.8 ± 3.7
Drakeol	7.5 ± 0.3	5.9 ± 0.2	6.5 ± 0.1	4.7 ± 0.3	145.5 ± 11.8	99.3 ± 14.2
Silica	7.4 ± 0.2	6.6 ± 0.2	8.0 ± 0.2	5.7 ± 0.4	150.5 ± 3.1	111.5 ± 15.5

[a] Groups of five mice were injected i.p. with drakeol or silica (see Table 4). 24 h later, the peritoneal cells were collected.

[b] Average cell counts ± standard error in millions per milliliter.

[c] Average cell count ± standard error per microscopy field of cells settled in 35-mm plastic petri dishes, to which 1 ml of resuspended cells and SRBCs had been added.

TABLE 6. *Effect of drakeol and silica on the LDV and HSV titers in the blood plasma and peritoneal wash fractions of mice*[a]

Titers[b]	Day 1			Day 3		
	Control	Drakeol	Silica	Control	Drakeol	Silica
LDV						
Blood plasma	9.9 ± 0.28	9.9 ± 0.42	9.9 ± 0.24	8.5 ± 0.20	7.5 ± 0.20	7.5 ± 0.20
Peritoneal wash	7.5 ± 0.20	8.3 ± 0.20	8.1 ± 0.28	7.3 ± 0.20	7.7 ± 0.20	8.3 ± 0.32
Centrifuge supernatant	7.5 ± 0.28	8.7 ± 0.20	8.9 ± 0.24	7.5 ± 0.20	7.7 ± 0.20	8.1 ± 0.24
Resuspended cells	<5.5	6.1 ± 0.24	6.9 ± 0.24	5.9 ± 0.24	5.9 ± 0.24	6.9 ± 0.24
HSV						
Blood plasma	0	0	0	0	0	0
Peritoneal wash	2.5	2.0	3.0	2.0	2.5	3.5
Centrifuge supernatant	3.0	2.25	>3.5	1.0	1.5	2.25
Resuspended cells	1.5	1.25	2.25	0.5	0.75	1.25

[a] See Table 4 for experimental conditions. Differences of ≥ 0.6 are significant at $P \leq 0.05$.

[b] LDV titers are expressed in $\log_{10}$ mean infective doses per milliliter ± standard deviation in sample. HSV titers are expressed in $\log_{10}$ $TCID_{50}$ per milliliter of sample.

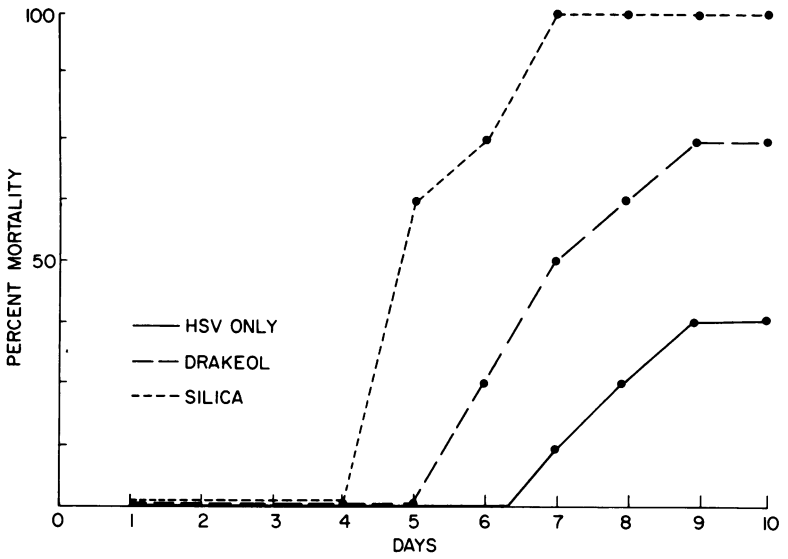

FIG. 2. *Effect of drakeol and silica on the percent mortality of mice infected with HSV. Day t_0: i.p. injection of 50 mg of silica or 1 ml of drakeol per mouse (eight mice per group). Day t_{+1}: all mice infected with HSV i.p., 10^5 $TCID_{50}$ per mouse.*

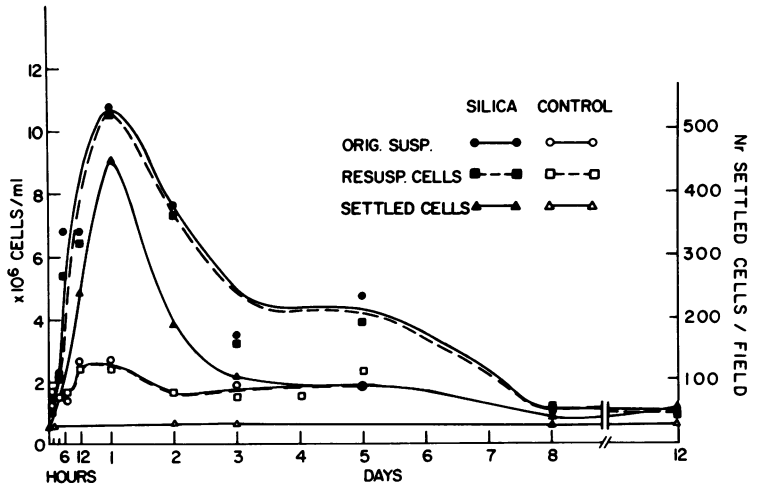

FIG. 3. *Duration of the effect of silica on the cell count of the peritoneal wash of mice. Mice were injected i.p. with 50 mg of silica per mouse. Cell counts at the time indicated on the abscissa. At each time period counts were made of the pooled washes (3.5 ml of MCM per mouse) of three mice. For further details see text.*

DISCUSSION

Intraperitoneal silica injections in mice have been reported to impair macrophage functions and thus allow viruses to spread rapidly to the target organs, which lead, as shown in the case of the HFEM strain of HSV, to an accelerated death of the silica-pretreated mice (2, 19). Since the VR_4 strain used here would not necessarily yield comparable results, experiments similar to those published previously were performed with this strain, using random bred female Swiss mice (Fig. 1). The results obtained with the two strains were indeed comparable. Whereas LDV, a model for slow viruses (11), appears to multiply exclusively in macrophages in vivo (4, 12, 13) as well as in vitro (5, 6), silica could, on the basis of the above described results, presumably be used to decrease the macrophage population and thus the LDV titer. As Table 1 shows, contrary to the above deductions, the LDV titer increased in vivo after silica

injection. This indicated either that LDV could multiply in other target cells or that the above deductions did not apply. As Tables 4 and 5 show, the number of glass-adherent cells, mainly macrophages, obtained from peritoneal washes of silica-injected mice increased, paralleling the increase in virus titer. Parallel experiments were done with a paraffin, drakeol (a nonsolid irritant with adjuvant effects similar to certain silicas [18]); these experiments showed that a comparable, but lower increase in cell counts occurred after i.p. injection of this material (Tables 4 and 5), and a corresponding increase in both LDV and HSV virus titers was found (Table 6). Apparently, the irritants supplied more target cells for the viruses, leading to higher virus titers. In the case of HSV the data do not exclude the additional effect of silica on other host cells for HSV. Pearsall and Weiser (9) also reported that silica treatment led to a decrease in peritoneal macrophages, followed by an increase, but in their case the decrease lasted for several days.

The above results support the interpretation that silica preparations with certain structural characteristics cause inflammatory reactions which lead to a rapid entry of blood monocytes which originate in the bone marrow (16). Such preparations can also act as adjuvants (18). It should be noticed that the reports on the inflammatory and adjuvant effects of silica vary widely. The most acceptable explanation is offered by Stanton and Wrench (15), who found that the effects of different silicas were related to their structural shape rather than to their physicochemical properties. For that reason an international standard sample of silica was used in this study. Our present results, together with those on the effect of superinfection with LDV on the virus titers in chronic LDV-infected mice (3), indicate that LDV might be an example of noncytopathic viruses, which, like silica, stimulate continuous emigration and proliferation of monocytes until the virus (again like the silica) becomes localized in a relatively few macrophages, with negligible proliferation and migration. It would be of interest to examine the effect of silica on chronic LDV and on other infections which might be induced to show recrudescence.

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