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Some Effects of Silica Treatment on Marek's Disease

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Treatment of newly hatched chicks with silica by the intraperitoneal route delayed the onset of mortalities due to the JM strain of Marek's disease (MD) virus inoculated at 6 days of age. During the 88-day observation period fewer silica-treated chicks died of MD, but this difference was not usually statistically significant. Silica treatment had no effect on the susceptibility of 4-week-old birds. Silica treatment reduced the antibody response to MD but, in general, not significantly. The antibody response to bovine serum albumin was significantly enhanced if measured by the indirect hemagglutination test but not if measured by the agar gel diffusion test, whereas the response to *Brucella abortus* was enhanced significantly in N-line (MD-resistant) chicks but not significantly in P-line (MD-susceptible) chicks. Five days after infection, silica-treated chicks had significantly less fluorescing antigen in thymus and bursa than did untreated chicks; no difference was observed in the spleen. After silica treatment the glass-adherent cell population in the buffy coat was increased by up to 10-fold compared with untreated chicks. It is suggested that silica treatment induced macrophage proliferation, with subsequent restriction of MD virus spread, yet allowed an adjuvant-type effect with other antigens.

Genetic resistance of fowls to Marek's disease (MD) is well recognized but the mechanism of resistance (or susceptibility) is not known. There is no difference in susceptibility to infection either of the host (7) or of cells from different hosts (27, 32). There is no fundamental difference between the humoral immune systems of susceptible and resistant birds (15). The tendency of susceptible lines to produce less antibody to MD virus is most appropriately attributed to the extensive lymphoid tissue damage occurring in these birds (14, 28, 30). Previous studies at this laboratory have suggested that the dichotomy between resistance and susceptibility occurs early in the pathogenesis of MD, probably before antibody production can play any significant part (7, 9). Genetic differences in thymus-dependent immune reactions have been examined in birds varying in genetic susceptibility to MD (8, 18), and theoretical consideration has been given to the thymus system as the mediator of genetic resistance (18, 23, 24); whereas the thymus system may be the origin of age-related resistance (29), there is no convincing evidence that it is the origin of genetic resistance.

The role of the reticuloendothelial system in pathogenesis and immunity of virus infections is not fully understood. Model disease systems in mice are, however, yielding much information in this direction. Macrophage reactivity seems to be the mechanism of age-related resistance of mice to herpes simplex virus encephalitis (16, 17) and of genetic resistance to murine cytomegalovirus (25). In spite of the ultimate death of macrophages infected with herpes simplex virus, they were considered to restrict replication of this virus (34). However, no difference could be observed in the replication of murine cytomegalovirus in macrophages from mice differing in susceptibility (25). It is tempting to draw parallels with the resistance to MD related to age and genetic strain and to postulate that the basis for such resistance might reside in the macrophage system.

We have attempted to examine the role of the reticuloendothelial system in pathogenesis of MD. Silica (silicon dioxide) is phagocytosed by macrophages and damages the phagosomes, resulting in release of hydrolytic enzymes into the cytoplasm and lysis (2). The cytotoxicity of silica seems quite specific for macrophages (21) and has subsequent effects on immune responsiveness (19). Here we describe some effects of silica on the incidence and pathogenesis of MD.

MATERIALS AND METHODS

Birds. N-line (MD-resistant) and P-line (MD-susceptible) (10) White Leghorn fowls were obtained from the departmental pathogen-free flock. Experi-

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ments were performed in a virus isolation building.

Silica. Silicon dioxide (fumed; particle size 0.05 μm, Sigma Chemical Co.) was washed three times in 0.1 M phosphate-buffered saline (PBS), pH 7.2, and recovered after each washing by centrifugation. It was reconstituted in PBS to 100 mg/ml and sterilized by autoclaving. Administration of silica was by the intraperitoneal route.

MD virus. Challenge was with 100 focus-forming units of the JM isolate (26) of MD virus injected into the breast muscle. Production and use of the challenge stock has been outlined elsewhere (14).

MD virus antibody assays. Virus neutralization (VN) and agar gel diffusion tests were performed as previously described (5).

Fluorescent antibody test. MD virus antigen was detected in sections of frozen thymus, spleen, and bursa of Fabricius by the direct fluorescent antibody test (9). The amount and distribution of antigen was scored 0 to 4 as previously described (6).

Brucella abortus. Each bird received 1.6×10^8 *B. abortus* strain 19 organisms in 0.1 ml of saline injected into the breast muscles. Antibody titers were assessed by the tube agglutination test (3) against a suspension of killed *B. abortus* and expressed as the reciprocal of the highest serum dilution giving 50% agglutination. (Antigens for inoculation and agglutination tests were kindly provided by J. R. Duncan.)

Bovine serum albumin. Bovine serum albumin (BSA) (bovine albumin, crystallized, Pentex, Miles Laboratories Inc.) was injected into the breast muscles as a 1% solution in saline at the rate of 5 mg/bird. Antibody to BSA was measured by the indirect hemagglutination test, using fowl erythrocytes sensitized with BSA by tannic acid (13), and by microimmunodiffusion (20) against 1.0% BSA in saline. Precipitin titers were expressed as the reciprocal of the highest serum dilution giving a visible precipitin line, and indirect hemagglutination titers were the reciprocal of the serum diluting giving 50% agglutination.

Quantitation of blood macrophages (glass-adherent cells). Two milliliters of blood was collected by cardiac puncture into sterile Alsever solution. The mixture was centrifuged in narrow tubes at about $800 \times g$ for 10 min. The white buffy coat was collected from the top of the column of erythrocytes and washed three times in sterile tissue culture medium, without added serum. The cells were then transferred to 9 ml of medium with 5% fetal bovine serum, mixed well, and pipetted into three plastic petri dishes, 35 mm in diameter. The dishes were incubated at 38 C in an atmosphere enriched with CO_2. Culture medium was changed daily, and the dishes were gently rinsed before the addition of new medium. After 7 days of culture, the dishes were drained of tissue fluid, rinsed with PBS, fixed with methyl alcohol, and flooded with Giemsa stain. Dishes were stained for 15 min and then rinsed with deionized water. The cells were counted in 12 high-power fields (magnification, ×120) for each dish, giving 36 field readings for each blood sample.

Statistical analysis. Group mortality figures were compared by the chi-square test for independence, antibody levels were compared by *t*-test, and fluorescing antigen scores and glass-adherent cell counts were each compared by analysis of variance (31).

Experimental design. Seven experiments were performed: five (1–5) used 1-day-old chicks and two (6 and 7) used 4-week-old chicks. The number of chicks in each experiment is reported in Results.

One-day-old chicks were treated with silica at the rate of 0.5 ml (50 mg)/dose on days 1 and 2 and 0.75 ml (75 mg) on day 4. Virus challenge was on day 6. Experiments 1 and 2 included the following treatments for each genetic line: (i) no silica, no virus; (ii) silica, no virus; (ii) silica, no virus; (iii) no silica, virus; (iv) silica and virus; (v) PBS (given in equal volumes to the silica) and virus. Mortalities were high among chicks receiving silica during and for some days after treatment; the number of birds in each experimental group was therefore taken as the number surviving 10 days after the termination of silica treatment. Birds were held for up to 88 days after virus challenge (94 days of age) to monitor the incidence of MD, which was confirmed by necropsy. At the end of each experiment sera from birds not challenged with MD were shown to be free of precipitating antibodies to MD virus, confirming the absence of adventitious infection.

Experiments 3 and 4 used treatment groups (i) through (iv) as above; experiment 3 used P-line chicks only, whereas experiment 4 examined N-line and P-line chicks. Chicks were killed on day 11 (5 days after virus challenge), and spleen, thymus, and bursa were examined by direct fluorescent antibody test for MD virus antigen.

In experiment 5 two groups of each genetic line were used. One group of each line was treated with silica; one group was untreated. On day 6, seven chicks from each group were exsanguinated for enumeration of glass-adherent blood cells. The remainder were inoculated with *B. abortus* and BSA. They were bled 3 weeks later, and sera were tested for antibodies.

The two experiments in 4-week-old birds commenced when the birds were 28 days old (day 1 of experiment). Silica treatment was at the rate of 2 ml (200 mg)/bird on days 1 through 5. Virus challenge was on day 6. The first of these two experiments (experiment 6) was designed to assess the effect of silica treatment on the incidence of MD. The experiment groups for each genetic line were as for experiments 1 and 2. Birds were held for 88 days after challenge, and incidence of MD was recorded.

In experiment 7, experiment groups (i) through (iv), as in experiment 1, were used. Birds were bled 6 days before and 10, 14, 17, and 21 days after challenge. Sera were examined in the VN and agar gel diffusion tests for antibodies to MD virus.

RESULTS

Effect of silica on incidence of MD. In experiments 1 and 2 fewer chicks died of MD in groups receiving silica than in groups not receiving silica (Table 1). Only one comparison (silica-treated versus PBS-treated in experi-

TABLE 1. *Incidence of MD mortalities in birds treated with silica or untreated before exposure to MD virus (experiments 1, 2, and 6)*

Expt	Age[a]	Genetic line	Treatment[b]	MD incidence[c] (dead/total [%])	
1	1	N	None	6/25 (24)	$P > 0.05$[d]
			Silica	1/20 (5)	
			PBS	4/20 (20)	$P > 0.05$
		P	None	7/24 (29)	$P > 0.05$
			Silica	1/13 (8)	
			PBS	9/18 (50)	$P < 0.05$
2	1	N	None	2/25 (8)	$P > 0.05$
			Silica	1/20 (5)	
			PBS	5/27 (19)	$P > 0.05$
		P	None	30/30 (100)	$P > 0.05$
			Silica	18/22 (82)	
			PBS	20/20 (100)	$P > 0.05$
6	28	N	None	3/30 (10)	$P > 0.05$
			Silica	1/39 (3)	
			PBS	0/30 (0)	$P > 0.05$
		P	None	22/25 (88)	$P > 0.05$
			Silica	22/24 (92)	
			PBS	21/25 (84)	$P > 0.05$

[a] Age of birds at day 1 of experiment.

[b] Silica or PBS treatment on days 1, 2, and 4 (experiments 1 and 2) and on days 1 to 5 (experiment 3). For doses, see text. Virus challenge on day 6.

[c] Incidence of MD expressed as number of birds dying of MD during 88-day period after virus challenge/number surviving 10 days after the end of silica treatment. Control birds (no silica, no virus; silica, no virus) did not show signs of MD and are not included in the table.

[d] Group values were compared by the chi-square test for independence. Interpretation of significance: $P > 0.05$, not significant; $P < 0.05$; possibly significant.

ment 1, P-line chicks) was possibly significant. Graphs of mortalities (Fig. 1) show that silica-treated chicks first suffered mortalities 10 to 14 days later than PBS-treated or untreated chicks. In experiment 6 there was no effect on incidence (Table 1) or time of occurrence of MD between silica-treated and untreated chicks. Summation of the data for each treatment in the three experiments, irrespective of genetic line, gave no significant difference between silica treatment and PBS treatment ($P > 0.05$) and only possible significance between silica treatment and no treatment ($P < 0.05$). In the three experiments there was a total of 17 birds of each line that were untreated, unexposed controls and 23 birds of each line that were silica treated but not challenged with MD virus. There were no mortalities in any of these groups.

Effect of silica on appearance of viral antigen in bursa, spleen, and thymus. In both experiments (3 and 4) the amount of viral antigen observed was reduced in thymus and bursa but essentially unchanged in the spleen of silica-treated chicks compared with nontreated chicks (Table 2).

Effect of silica on blood macrophage counts. Silica-treated chicks in experiment 5 had many more glass-adherent cells than untreated chicks (Table 3).

Effect of silica on production of antibody to *B. abortus* and BSA. In experiment 5 silica-treated chicks had higher mean antibody titers to both antigens than did nontreated chicks, although not all of the differences were statistically significant (Table 4).

Effect of silica on production of antibody to MD. The results of experiment 7 (Table 5) show that VN antibodies were present by 10 days after challenge. At this time there was no significant difference in incidence or titers of antibody either between lines or treatments. However, by 21 to 28 days postinfection, N-line birds had more VN antibody than P-line birds, and silica-treated birds of each line had less VN antibody that nontreated birds. Statistical comparison of the silica-treated versus untreated birds of each line showed significant difference only on one occasion, i.e., in the N-line birds bled 28 days after challenge.

DISCUSSION

After challenge of neonatal chicks, quite high mortalities were observed in both the N (MD resistant)- and P (MD susceptible)-lines, although the P-line chicks were in general more susceptible than the N-line chicks. The N-line mortalities were consistent with observations that so-called genetically resistant birds without passively acquired antibodies and prior to the development of age-associated resistance are susceptible to MD (7). Treatment of neonatal chicks with silica had a statistically insignificant effect on their susceptibility to MD, yet a consistent trend was seen towards survival in the silica-treated groups. The results suggested that this trend could be due either to an absolute and permanent (i.e., for at least the duration of the experiment) reduction in susceptibility or to delay of up to 14 days in the onset of mortalities, perhaps due to effects of the silica treatment on early pathogenesis.

Treatment of 4-week-old birds with silica failed to affect their susceptibility to MD. It was noteworthy that in 4-week-old birds silica treat-

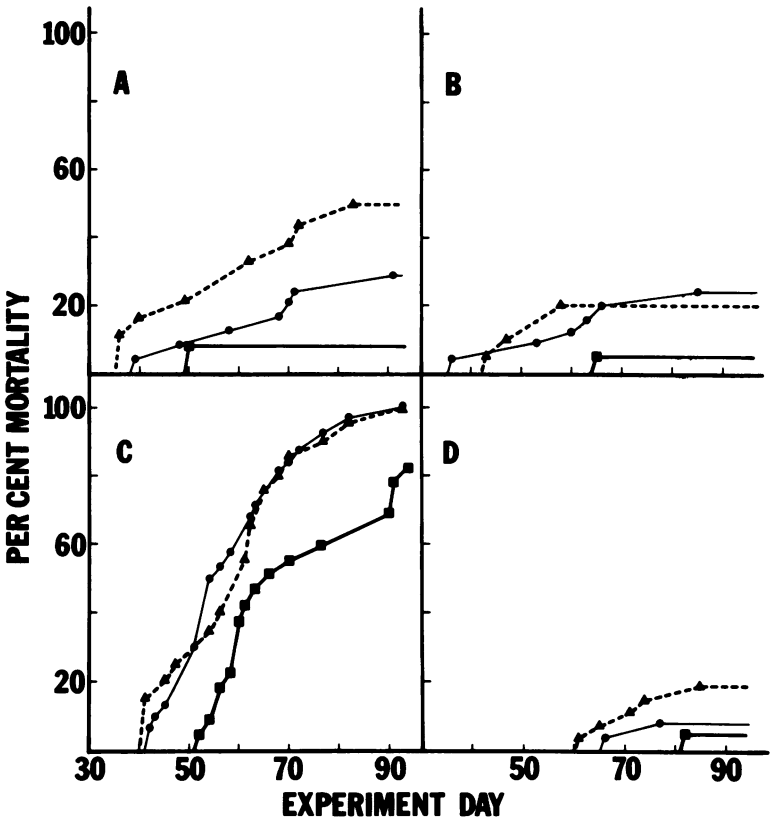

FIG. 1. *Cumulative percent mortality curves in two experiments to assess the effect of silica treatment on the susceptibility of chicks to MD. (A) Experiment 1, P-line chicks; (B) experiment 1, N-line chicks; (C) experiment 2, P-line chicks; (D) experiment 2, N-line chicks. Symbols: ▲, PBS-treated birds; ■, silica-treated birds; ●, untreated birds. Experiment day 1 was day of hatch. Silica or PBS treatment was on days 1, 2, and 4. Virus challenge was on day 6.*

TABLE 2. *Effect of silica treatment of newly hatched chicks on amount of MD virus antigen (detected by fluorescent antibody [FA] test) in spleen, bursa, and thymus 5 days after virus challenge (experiments 3 and 4)*

Expt	Genetic line	No. of birds	Silica treatment[a]	FA test score[b]					
				Thymus		Spleen		Bursa	
3	P	10	No	62.5	$P < 0.05$[c]	55.0	$P > 0.1$	45.0	$P < 0.05$
	P	10	Yes	25.0		57.5		17.5	
4	N	27	No	35.2	$P < 0.05$	37.0	$P > 0.1$	31.5	$P < 0.01$
	N	33	Yes	23.5		35.4		9.8	
	P	30	No	41.7	$P > 0.05$	38.9	$P > 0.1$	27.8	$P < 0.01$
	P	35	Yes	30.0		41.4		6.4	

[a] Silica treatment on days 1 (50 mg), 2 (50 mg), and 4 (75 mg), followed by virus challenge on day 6.

[b] FA score was determined on tissues collected on day 11. The FA result was scored for each tissue, 0–4 (according to Calnek, reference 5), and the scores of that tissue for the group were summed. The score was then expressed as a percentage of the possible maximum. For example, if the sum score for 10 spleens is 23, since the maximum possible score is 40 the group percentage score is 57.5. In each experiment five birds of each line (P in experiment 3, N and P in experiment 4) were treated no silica/no virus and silica/no virus; all FA tests on these chicks were negative.

[c] Group values were compared by analysis of variance. Interpretation of significance: $P > 0.1$, $P > 0.05$, not significant; $P < 0.05$, possibly significant; $P < 0.01$, significant difference.

ment had little systemic effect and caused only few deaths. In neonatal chicks, however, silica injections caused many deaths, and surviving chicks appeared weak and lethargic. The stress of silica treatment in young chicks might therefore have played some part in reduction of MD mortalities, as has been reported in other stress situations (11).

TABLE 3. *Blood macrophage (glass-adherent cells) counts in buffy-coat cultures from control and silica-treated N-line and P-line chicks (experiment 5)*

Line	No. of birds	Silica treatment[a]	Macrophage counts ± standard deviation[b]	
N	7	No	57.63 ± 10.91	$P < 0.01$[c]
N	7	Yes	181.47 ± 45.27	
P	7	No	22.75 ± 5.08	$P < 0.01$
P	7	Yes	384.01 ± 63.61	

[a] Silica treatment days 1 (50 mg), 2 (50 mg), and 5 (75 mg).

[b] The mean count per field was determined for each of the seven birds in a group by counting and averaging a total of 36 fields from three buffy-coat cultures. The overall group mean counts and standard deviations reported in the table were calculated from the seven mean values in each group, not from the individual field readings.

[c] Group values were compared by analysis of variance. Interpretation of significance: $P < 0.01$, significant difference between groups.

Direct fluorescent antibody tests on thymus, spleen, and bursa, performed at a time of marked infection of these organs (1), showed less viral antigen in thymus and bursa of silica-treated birds than in untreated birds. Antigen content of the spleen was unaffected. The most reasonable explanation of this observation is that the spread of virus from the site of injection (pectoral muscles) to the spleen occurs by a different mechanism or pathway from the spread to thymus and bursa, and that this latter pathway was selectively affected by the silica treatment.

Since neonatal chicks elicit poor antibody responses to MD virus, 4-week-old birds were used to assess the effect of silica treatment on production of anti-MD antibody. Production of VN antibody followed the biphasic pattern previously described (14). Silica treatment reduced the amount of antibody in the second peak (14 or more days after virus challenge) but not in

TABLE 4. *Mean reciprocal antibody titers to B. abortus and BSA in N- and P-line chicks, treated and untreated with silica, 3 weeks after inoculation at day 6 (experiment 5)*

Genetic line	Silica treatment[a]	Antibody titers[b] (no. with antibody/no. in group; arithmetic mean) against:					
		B. abortus		BSA			
				IHA[c]		AGP[c]	
N	No	8/10; 14	$P < 0.01$[d]	10/10; 89.6	$P < 0.01$	8/10; 2.0	$P > 0.05$
N	Yes	8/10; 244		10/10; 921.6		7/10; 3.4	
P	No	8/10; 48	$P > 0.05$	10/10; 75.2	$P < 0.01$	6/10; 2.0	$P > 0.05$
P	Yes	10/10; 56		10/10; 1,127.2		10/10; 7.2	

[a] Silica treatment days 1 (50 mg), 2 (50 mg), and 4 (75 mg).

[b] For antibody assays see text. Means are expressed as the arithmetic mean of the reciprocal titers for the group.

[c] IHA, Indirect hemagglutination test; AGP, microimmunodiffusion.

[d] Group values were compared by t test. Interpretation of significance: $P > 0.5$, not significant; $P < 0.01$, significant difference.

TABLE 5. *Incidence and levels of VN antibody in N-line and P-line birds treated daily (200 mg/dose) with silica or not treated at 28 to 32 days of age and challenged with MD virus at 33 days of age (experiment 7)*

Genetic line	Silica treatment	No. of birds (of 10) with antibody (mean titer) at postchallenge day:[a]									
		10		14		17		21		28	
N	No	7 (24)	$P > 0.05$[b]	4 (10)	$P > 0.05$	5 (18)	$P > 0.05$	8 (24)	$P > 0.05$	10 (32)	$P < 0.01$
N	Yes	10 (30)		1 (2)		5 (5)		5 (12)		5 (10)	
P	No	10 (24)	$P > 0.05$	3 (10)	$P > 0.05$	3 (5)	$P > 0.05$	3 (10)	$P > 0.05$	4 (10)	$P > 0.05$
P	Yes	9 (25)		7 (15)		0 (0)		5 (5)		3 (3)	

[a] Mean antibody titer expressed as the arithmetic mean (10 birds) of the highest serum dilution giving 50% VN against 100 focus-forming units of virus. All birds were bled 6 days before challenge, and sera gave negative results at that stage. Ten birds of each line untreated with silica and unchallenged were bled at 28 days after challenge of experimental groups; these two were negative for MD virus antibody.

[b] Group values were compared by t test. Interpretation of significance: $P > 0.05$, not significant; $P < 0.01$, significant difference.

the first peak (10 days after challenge). Lower antibody levels in the silica-treated birds are consistent with the parallel observation that silica treatment of neonatal chicks reduced the levels of viral antigen in the bursa and thymus.

Silica treatment of mice renders them more susceptible to both herpes simplex virus (36) and murine cytomegalovirus (25). Recent studies with lactic dehydrogenase virus and herpes simplex virus demonstrated a proliferation of macrophages after treatment of mice with silica (4). This is similar to the present study, in which it seems a quantitative enhancement of the reticuloendothelial system was responsible for subsequent effects on pathogenesis and antibody production. Thus, an increase in macrophage population would result in restriction of MD virus spread (assuming that macrophages can restrict the replication of MD virus), with less rapid and perhaps fewer subsequent mortalities and lower antibody response due to the lesser amount of MD antigen available to the host, yet an adjuvant effect in terms of response to BSA and *B. abortus*. This is compatible with studies in mammals showing an adjuvant effect of silica and aluminum silicate (22, 35); silica-treated rats and rabbits produced much more antibody to horse serum and ovalbumin, respectively, than untreated animals, and histological studies suggested that a cellular proliferation in the reticuloendothelial system was related (22).

Further examination of the dynamics of the cell populations after silica treatment is required. "Normal" populations are themselves quantitatively highly variable. The response to irritants and toxins fluctuates rapidly in terms of quantitative cellular response (12). The many systems of silica treatment described in the literature and the divergent results obtained suggest that such factors as dose, route of inoculation, and timing relative to antigen administration can make the difference between immunosuppression and enhancement. The variation in effect of different forms of silica might be attributable to the shape of the silica particles (33).

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