Inhibition *in vivo* of Reticulo-Endothelial Phagocytosis by Synthetic Polyamino Acids.* (28664)

KURT STERN
Department of Pathology, University of Illinois College of Medicine, Chicago

We reported(1) that administration of synthetic polylysine (PL) to mice significantly depressed formation of antibodies for sheep red cells. This effect was not mediated by damage to lymphoid tissues, nor did it depend on direct interaction of PL with antigens of the red cell surface, since sheep red cells treated *in vitro* with PL prior to injection into mice induced antibody formation at least as well as did untreated red cells. In view of the low molecular weight (approximately 2,600) and the small total effective dose (less than 2 mg per mouse), it was also unlikely that storage of the compound in reticulo-endothelial (r.e.) cells produced a "blocking" effect. The possibility was considered that the diminished antibody response may have resulted from an inhibition of phagocytosis of red cells by r.e. cells. This paper presents experimental evidence for such an effect of PL as well as of synthetic polyglutamic acid (PG). A preliminary report of some of these findings has been made(2).

Materials and methods. Animals. Healthy, 3 to 5 months old mice, raised in our laboratory from inbred strains BALB/c/S, C3H/S, ASW/S, and DBA/S, and F_1 mice derived from the inbred strains, were used.

Polyamino acids. Polylysine hydrochloride and polyglutamic acid were kindly made available by the Kremers Urban Co., Milwaukee. Both polyamino acids were stated to have average chain lengths of 20. Stock solutions of 1% polyamino acid in 0.9% NaCl were adjusted to pH 7 with dilute NaOH in the case of PL, and with dilute HCl in the case of PG. The solutions were kept under refrigeration. On the day of use, the stock solution was diluted 10 times with 0.9% NaCl so that the dilute solutions used for injection contained 1 mg of the polyamino acid per ml.

Labeling of sheep red cells. This procedure was carried out immediately before injection of the red cells. After washing sheep red cells 3 times each with 10 volumes of 0.9% NaCl, 1 ml of packed red cells was well mixed with 0.5 ml of $NaCr^{51}O_4$† diluted with 0.9% NaCl to contain 12 to 20 μc/ml. After incubation at room temperature for 30 minutes, the red cells were washed 3 times each with 5 ml of 0.9% NaCl. The last washings contained only significant amounts of radioactivity. In most experiments, the efficiency of labeling ranged from 75 to 85%. The activity of a 10% suspension in 0.9% NaCl of labeled red cells varied from 0.5 to 0.8 μc/ml.

Experimental design. For each experiment, mice of the same strain, matched as to sex, age, and weight were divided into control and experimental groups. Intraperitoneal injections of 0.3 ml of the dilute polyamino acid solution (0.3 mg) were given twice daily to experimental animals, while control mice received at the same time 0.3 ml of 0.9% NaCl intraperitoneally. Three to 5 such injections were given on successive days. Three to 5 hours after the last polyamino acid or NaCl injection, all mice were injected intraperitoneally with 0.5 ml of a 10% suspension of the Cr^{51}-labeled sheep red cells. The skin puncture of this injection was sealed with collodion simultaneously with the withdrawal of the needle, to prevent leakage of the injected material. All animals were weighed and killed with ether 24 hours after injection of sheep red cells; livers and spleens were immediately removed, weighed, and preserved for radioassay.

Modifications of this basic experimental design when used, will be described in connection with the results of pertinent experiments.

Radioassay. Aliquots of 1 ml of suitable dilutions of the labeling solution and of the

* Supported in part by grants from Am. Cancer Society, Illinois Division, Inc., and Nat. Science Foundation.

† Squibb Chromitope Sodium, 100 μc/ml.

TABLE I. Phagocytosis of Sheep Red Cells in Polylysine (PL)-Treated Mice.

Strain	Sex	Group	No. of mice	No. of PL inj	Liver: Mean % body wt	Liver: Mean % uptake of injected cpm/g ± S.D.	Spleen: Mean % body wt	Spleen: Mean % uptake of injected cpm /100 mg
C3H	♂	Control	10	—	5.64	24.25 ± 6.02*	.86	1.94‡
		Exp	10	5	5.41	11.49 ± 4.40	.68	.98
C3H	♂	Control	4	—	5.60	30.47 ± 8.06*	.94	2.48*
		Exp	5	5	5.56	5.73 ± 2.70	.61	.75
C3H	♀	Control	5	—	5.21	33.05 ±11.41‡	.71	4.58†
		Exp	5	5	5.24	7.45 ± 2.51	.91	1.11
BALB/c	♂	Control	9	—	6.53	19.66 ± 6.65*	1.09	1.61
		Exp	9	6	6.33	11.72 ± 7.44	.94	1.90
BALB/c	♀	Control	7	—	6.18	22.04 ± 4.29*	1.06	1.56‡
		Exp	14	4	6.09	10.45 ± 4.80	.91	.67
ASW	♂	Control	8	—	5.67	12.80 ± 6.36‡	.51	.73
		Exp	10	5	5.03	6.68 ± 4.74	.60	.53
ASW	♂	Control	8	—	6.03	17.24 ± 3.15*	.56	3.85*
		Exp	8	4	5.75	5.12 ± 1.61	.48	.68
ASW	♂	Control	5	—	5.51	25.97 ± 4.79‡	.78	2.35
		Exp	5	3	5.54	8.17 ± 1.19	.75	2.20
ASW	♀	Control	5	—	5.67	30.06 ± 2.58*	.60	3.44†
		Exp	5	3	4.97	5.92 ± 2.34	.68	1.17

Significance of difference in uptake of control and experimental groups:
* $P < .001$. † $P < .01$. ‡ $P < .05 > .01$.

suspension of labeled red cells were placed into plastic tubes and assayed in a well scintillation counter.‡ The efficiency of the counter was determined as roughly 5%, with 1 μc of Cr^{51} yielding approximately 110,000 CPM. At termination of the experiments, the livers and spleens were placed into plastic tubes for radioassay in the well counter. After correction for decay, the uptake of Cr^{51} was determined for liver and spleen as per cent of injected CPM which was present in the whole organ, and calculated for the unit of weight (g of liver; 100 mg of spleen). When differences in uptake of labeled red cells were observed in control and experimental groups, their statistical significance was determined by means of Student's *t* test.

Results. Body weights determined at the beginning and end of the experiments did not show significant differences between control and experimental animals. Administration of polyamino acids at the dose levels described did not produce obvious signs of toxicity, nor were gross abnormalities noted on autopsy of the treated mice.

‡ RIDL Scintillation Counter, model 60-2; 11-1 well with 1¾ x 2″ crystal.

Table I summarizes data from 9 experiments in which phagocytosis of sheep red cells was compared in control and PL-injected mice. This treatment significantly depressed hepatic phagocytosis in all experiments, and splenic phagocytosis in 6 out of 9. Differences in hepatic and splenic weight between control and experimental mice did not appear to be correlated with this interference with phagocytosis.

Table II contains the results of an experiment in which female ASW mice were divided into 3 groups: experimental mice of Group I received a total of 3 injections, or 0.9 mg of PL; those of Group II, 5 injections, or 1.5 mg; those of Group III, 12 injections, or 3.6 mg. While phagocytosis was depressed in all groups, the ratios between uptake in control mice and that in experimental mice (C/E) showed that the effect on hepatic phagocytosis was directly proportional to the dose of PL used. Splenic phagocytosis was inhibited to a greater degree by 1.5 mg than by 0.9 mg, but 3.6 mg was less effective than 0.9 mg.

TABLE II. Effect of Dose of Polylysine (PL) on Phagocytosis (ASW Female Mice).

Group	No. of mice	No. of PL inj	Liver: Mean % body wt	Liver: Mean % uptake of injected cpm/g ± S.D.	Liver: C/E*	Spleen: Mean % body wt	Spleen: Mean % uptake of injected cpm/100 mg	Spleen: C/E*
I Control	5	—	4.69	36.45 ± 1.36†	1.75	.46	8.64‡	3.25
Exp	5	3	4.60	20.92 ± 4.36		.47	2.67	
II Control	5	—	6.31	18.87 ± 3.94†	2.58	.50	2.77†	5.45
Exp	5	5	5.83	7.32 ± 3.93		.74	.51	
III Control	6	—	5.96	34.86 ± 4.91†	5.15	.72	2.95†	3.30
Exp	6	12	6.58	6.78 ± 2.95		.65	.89	

* Uptake in controls/uptake in experimental mice.

Significance of difference in uptake of control and experimental groups:
† $P < .001$. ‡ $P < .05 > .01$.

TABLE III. Duration of Depression of Phagocytosis Caused by Polylysine.

Group*		Injections: Days	Injections: No.	Inj of Cr^{51}-sheep RBC on day	Liver: Mean % uptake, g	Spleen: Mean % uptake, 100 mg
I	Control	—	—	4	21.58†	6.46†
	Exp	1 - 4	7		7.54	.79
II	Control	—	—	11	20.84	4.28‡
	Exp	1 - 4	7		16.70	1.57
III	Control	—	—	18	23.52†	2.00‡
	Exp (a)	1 - 4	7		6.66	.63
	(b)	16 -18	5			

* Each group consisted of 4 female (C3H × BALB/c)F_1 mice.
† $P < .001$.
‡ $P < .01$.

One of several experiments undertaken in order to investigate the duration of the effect of PL is presented in Table III. Mice of experimental Groups I, II, and III received 7 injections of PL on 4 successive days, while control mice were injected with 0.9% NaCl. All mice of Group I were injected intraperitoneally with labeled sheep red cells 5 hours after the last injection of PL of NaCl, in accord with the procedure used in previous experiments. The expected depression of hepatic and splenic phagocytosis occurred. On the other hand, when mice of Group II were injected with labeled sheep red cells 7 days after the last PL injection, hepatic phagocytosis was not affected, and splenic phagocytosis only slightly. Mice of Group III were given a second series of 5 injections of PL 12 days after the last injection of the first series. When phagocytic uptake was tested in these mice by injecting labeled sheep red cells given intraperitoneally 5 hours after the last PL or NaCl injection, the depression of phagocytosis was comparable to that observed in treated mice of Group I.

Since the initial observations(1) on interference with formation of antibodies for sheep red cells in PL-treated mice were made after a course of 6 immunizing injections, the effect of PL on phagocytic uptake after multiple injections of labeled sheep red cells was also tested. DBA female mice, divided into control and experimental groups, received during a period of 2 weeks 6 intraperitoneal injections each consisting of 0.3 ml of a 5% suspension of Cr^{51}-labeled sheep red cells. On the day before each red cell injection, control mice were injected intraperitoneally with 0.3 ml of 0.9% NaCl and experimental mice with 0.3 ml of the 0.1% PL solution. All animals were killed 24 hours after the last red cell injection, and hepatic and splenic uptakes were determined by radioassay, with the calculations taking into consideration the cu-

TABLE IV. Effect of Polylysine on Phagocytosis After Multiple Injections of Sheep Red Cells (DBA Female Mice).

			Liver		Spleen	
Exp	Group	No. of mice	Mean % body wt	Mean % uptake of injected cpm/g ± S.D.	Mean % body wt	Mean % uptake of injected cpm/100 mg
I	Control	9	6.69	4.81 ± 1.62*	.53	.56
	Exp	10	6.66	2.12 ± 1.14	.55	.34
II	Control	10	6.28	7.74 ± 1.52*	.66	.67
	Exp	24	6.10	2.38 ± .95	.55	.86†

* $P < .001$. † See text for qualifying comment.

TABLE V. Phagocytosis of Sheep Red Cells in Polyglutamic Acid-Injected Mice.

				Liver		Spleen	
Strain	Sex	Group	No. of mice	Mean % body wt	Mean % uptake of injected cpm/g ± S.D.	Mean % body wt	Mean % uptake of injected cpm/100 mg
ASW	♂	Control	8	6.03	18.59 ± 3.15*	.56	3.85*
		Exp	7	5.83	6.07 ± 3.74	.39	1.11
ASW	♂	Control	5	5.40	15.77 ± 4.10†	.62	2.94‡
		Exp	5	5.50	3.54 ± .79	.62	.87
C3H	♂	Control	4	5.60	30.47 ± 8.26†	.94	2.32†
		Exp	5	4.89	12.00 ± 4.10	1.07	1.16
C3H	♀	Control	5	5.21	33.05 ±11.41†	.71	4.55†
		Exp	5	5.75	12.10 ± 4.81	.82	1.15
BALB/c	♂	Control	5	4.92	23.68 ± 7.32‡	.85	3.06
		Exp	5	5.73	8.92 ± 7.22	.82	1.68

Significance of difference between control and experimental groups:
* $P < .001$. † $P < .01$. ‡ $P < .05 > .01$.

mulative dose and radiodecay of the red cells administered. Two such experiments disclosed significant depression of hepatic phagocytosis in PL-treated mice (Table IV). In Exp. II, only 4 experimental mice exhibited detectable levels of radioactivity in the spleen, while spleens of the remaining 20 mice yielded counts below the threshold of sensitivity of the instrument; hence splenic phagocytosis was also depressed.

Mice which received 3 intraperitoneal injections, each of 0.3 mg of PG, prior to administration of labeled sheep red cells, exhibited a significant depression of hepatic phagocytosis in all experiments; splenic phagocytosis was depressed in 4 of the 5 experiments (Table V).

Discussion. The data presented have unequivocally demonstrated that treatment of mice with rather small amounts of some synthetic polyamino acids interferes with phagocytosis of heterologous red cells in the liver and, somewhat less consistently, in the spleen. For PL this effect was shown to be to some extent dose-dependent. The inhibition was temporary, inasmuch as phagocytosis returned to an almost normal range when 7 days had elapsed between the last PL injection and the injection of sheep red cells. When mice were treated with 2 series of PL injections, they responded to the second series with a depression of phagocytosis comparable to that seen in mice given only one series. Thus there was no indication for development of "tolerance" to the polyamino acid.

It is reasonable to hold this inhibition of reticulo-endothelial phagocytosis responsible for the previously reported decrease in formation of antibodies in PL-treated mice immunized with sheep red cells(1). In these experiments the depression of immune responses occurred more frequently in male than in female mice. This sex difference may reflect the fact that female mice of some strains are superior to males in ability of

forming immune antibodies to sheep red cells (3). Stimpfling(4) also found female C57BL mice to possess higher titers of *natural* agglutinins for sheep red cells than male mice. Hence, female mice might be able to respond with an adequate antibody formation even when treatment with polyamino acids has reduced the amount of phagocytosed antigen.

De Vries and associates(5) reported that PL promoted phagocytosis of starch granules by polymorphonuclear leukocytes *in vitro*. Buchanan-Davidson and co-workers(6) found that *in vitro* phagocytosis by leukocytes of Pneumococci was enhanced by PL, while phagocytosis of *Staphylococcus aureus* or of *Streptococci* was not affected. No meaningful comparison between these observations and our findings appears possible because of the profound differences in test conditions: *in vitro vs in vivo;* cellular elements: polymorphonuclear leukocytes *vs* tissue macrophages; and substrates: starch granules or bacteria *vs* red cells.

No explanation can be offered for the mechanism of the interference with phagocytosis brought about by the polyamino acids. The cationic nature of PL cannot be considered to be a critical factor, since the polyanionic PG had a similar effect. A direct "blocking" effect on reticulo-endothelial cells is unlikely because of the small molecular size and low effective dose of the polyamino acids. Kornguth, Stahmann and Anderson (7) demonstrated adsorption of fluorescent polylysine to the lipoprotein component of the membrane of Ehrlich ascites carcinoma cells. Although only minimal amounts of the compound appeared to penetrate into the cytoplasm, severe disorganization in mitochondrial and nuclear structure of the cells was detected. Conceivably, similar surface binding of polyamino acids could result in structural and functional disturbances of reticulo-endothelial cells and account for the effects reported. Another mechanism is suggested by the observation that precipitates are formed when PL is added *in vitro* to mouse serums in concentrations and quantities comparable to those used in the *in vivo* experiments. Thus one might consider the possibility that similar precipitates are produced in the animal injected with polyamino acid. The insoluble complex, by undergoing phagocytosis, may be responsible for "blocking" the uptake of some other particulate substrate, such as the sheep red cells. This hypothesis is being tested.

Summary. 1. Treatment of mice with synthetic PL or PG regularly inhibited hepatic, and frequently also splenic, phagocytosis of subsequently injected Cr^{51}-labeled sheep red cells. 2. This inhibition was roughly proportional to the dose of polyamino acid, and disappeared after approximately one week. 3. The interference with phagocytosis was offered as an explanation for the previously reported depression of formation of antibodies in mice treated with PL.

1. Stern, K., *J. Immunol.,* 1960, v84, 295.

2. ———, *Fed. Proc.,* 1963, v22, 672.

3. Stern, K., Davidsohn, I., *J. Immunol.,* 1955, v74, 479.

4. Stimpfling, J. H., *ibid.,* 1960, v85, 530.

5. De Vries, A., Salgo, J., Matoth, Y., Nevo, A., Katchalski, E., *Arch. internat. pharmacodyn.,* 1955, v104, 1.

6. Buchanan-Davidson, D. I., Seastone, C. V., Stahmann, M. A., *J. Bact.,* 1960, v80, 590.

7. Kornguth, S. E., Stahmann, M. A., Anderson, J. W., *Exp. Cell. Res.,* 1961, v24, 484.

Received June 27, 1963. P.S.E.B.M., 1963, v114.