Indomethacin Inhibits the Local Shwartzman Reaction (34751)

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When a rabbit receives an intradermal injection of a bacterial endotoxin and a day later receives another dose of endotoxin by the intravenous route, a hemorrhagic spot appears at the dermal site; the phenomenon is known as the local Shwartzman reaction. The infiltration of polymorphonuclear cells which follows the preparatory injection is an essential feature of the reaction.

Phelps and McCarty (1), using the Boyden chamber, showed that the migration of polymorphonuclear leukocytes across a membrane in response to a chemotactic influence can be inhibited by indomethacin. They also found that leukocytes taken from one dog dosed with indomethacin did not migrate at normal rates. A good question is whether such inhibition of migration *in vitro* is actually important in the body. If it is, one would suppose that indomethacin would inhibit the Shwartzman reaction to some degree, provided that it be given early enough to prevent the leukocytic infiltration caused by the preparatory injection. If the drug is delayed until the second, or eliciting, injection, one would expect little or no effect. The experiments to be described were therefore designed to show whether indomethacin does inhibit the reaction, and what may be the influence of timing of the drug dosage. Indomethacin does indeed inhibit, and the time relationships are as we have conjectured. On the other hand, indomethacin did not prevent the polymorphonuclear cells from gathering at the site of injection. One is forced to conclude that the drug must have prevented the cells from carrying out their usual enzymic processes, probably by preventing the lysosomes from leaving the cell and thereby becoming activated, or by preventing action of lysosomal enzymes within the phagosome formed as a result of contact of the cell with the object to be phagocytized.

Methods. Six normal rabbits and six treated with indomethacin were used in each of six experiments. Control and treated rabbits occupied alternate cage positions in the rack so as to equalize any possible effects of air flow and temperature; they were injected and examined in order of cage position. The endotoxin was from Difco Laboratories; *E. coli* serotype 026:B6 was used for one preliminary eperiment and 0127:B8 for the others, with no difference being obvious. The rabbits weighed from 2.4 to 3.6 kg, and in each experiment treated and control animals were selected so as to have equal weights.

The rabbits were shaved on the abdomen, and 4 intradermal preparatory injections of 50 μg each (0.5 ml each, in 0.9% sodium chloride solution) were made in each rabbit. Twenty to 24 hr later, the eliciting injections of 100 μg were made intravenously with the same solution, 1 ml/rabbit. These details were purposely identical with those used by Thomas (2) and by Halpern (3), except that we used four injection sites rather then one. Indomethacin, 450 mg, was dissolved with gentle heat in 5 ml of Emulphor EL-620 (polyoxyethylated vegetable oil, General Aniline Co.); this solution was then diluted to 100 ml, using 0.9% sodium chloride solution. It was injected subcutaneously, 9 mg/kg at each dose, in a volume of 2 ml/kg of body weight. We gave indomethacin in the odd-numbered (late) experiments at 1 hr before the eliciting endotoxin and 6 hr after it, or in the even-numbered (early) experiments at 1 hr before the skin injections, 6 hr after them, 1 hr before the eliciting endotoxin, and finally at 5 hr after it. Control rabbits received the same volume (2 ml/kg) of saline–Emulphor solution.

Each spot was measured (mm) in its lon-

gest direction and at right angles thereto; the product of these measurements roughly approximated the area. This value was then multiplied by a value from 0 to 4 representing the depth of color, as follows: 0 = no detectable response (cream-colored spots were not counted); 1 = pinkish; 2 = definite red; 3 = deep red; and 4 = mainly black. Most lesions were not uniform in color or regular in shape, and individual judgment had some influence.

In a separate experiment, six rabbits received indomethacin subcutaneously, 9 mg/kg, and 1 hr later were given endotoxin intradermally as in the previous work. Six other rabbits were given endotoxin only. Twenty-three hr later, all rabbits were sacrificed and the injected sites, including skin, subcutaneous tissues, and underlying abdominal muscles were fixed in Bouin's solution. Hematoxylin- and eosin-stained paraffin sections were examined microscopically for the presence of inflammation and particularly for the infiltration of polymorphonuclear leukocytes. The samples were coded so that the persons preparing and evaluating the sections had no knowledge of which rabbits had received treatment. The amount of polymorphonuclear infiltration was graded by the following system: ½ = trace; 1 = very slight; 2 = slight; 3 = moderate; and 4 = marked. Each of the four lesions on any rabbit was graded, and the sum of the four numbers was taken as the numerical result for that rabbit.

Results. The sites of intradermal injections showed cream-colored or pinkish tinges at 3 hr after the eliciting injection, but the variability was larger than with the more definite readings taken at 6 and 24 hr. In general, the best readings were at 24 hr, although some spots already may have begun to fade slightly.

In a preliminary experiment with only 4 control and 3 treated rabbits, indomethacin was given at the time of the first endotoxin injection and 6 hr later, and also at the time of the eliciting injection. Six hr later, scores (means per rabbit, *i.e.*, sums of 4 spots each) were 5902 for control and only 11 for treated rabbits. This result was encouraging enough so that we then performed six experiments shown in Table I. Values recorded by both observers are shown so as to illustrate the reproducibility of independent observations.

TABLE I. Effect of Indomethacin in Schwartzman experiments, observed at 24 hr.

Number of rabbits is shown in parentheses. Scores were calculated as described in Methods section, and represent the mean per rabbit of the sums of the four spots on each rabbit.

Exp. no.	Time of dosage	Observer	Control	Treated	Mean, treated (as % of control)[a]
1	Late	CGVA	6260 (6)	6032 (6)	96
		RPC	8170 (6)	7867 (6)	
2	Early	CGVA	4040 (4)	336 (6)	9
		RPC	4935 (4)	541 (6)	
3	Late	CGVA	834 (6)	645 (6)	63
		RPC	1022 (6)	497 (6)	
4	Early	CGVA	899 (5)	108 (6)	15
		RPC	1095 (5)	199 (6)	
5	Late	CGVA	5737 (5)	4117 (6)	70
		RPC	6501 (5)	4504 (6)	
6	Early	CGVA	3181 (6)	0 (6)	0
		RPC	2528 (6)	0 (6)	

[a] Means of treated rabbits as percentage of control: Early treatment 8%; Late treatment 76%; for statistical treatment see text.

In the even-numbered (early) experiments, in which we gave indomethacin at 1 hr before the preparatory endotoxin and 6 hr after it, also at 1 hr before the eliciting endotoxin and 5 hr after it, the mean 24-hr score was 197 ($n = 18$). In the odd-numbered (late) experiments, in which we gave indomethacin at 1 hr before the eliciting endotoxin and 6 hr after it, the mean score was 3943 ($n = 18$). For all control rabbits, the mean score was 3735 ($n = 32$). The 24-hr means expressed as a percentage of the control values within each experiment were 8% of the early treatment and 76% for the later treatment.

We found, rather surprisingly, that one could arrive at identical conclusions about early versus late treatments by ignoring the depth of color and merely measuring the area of the spot. The cube root of the first parameter (length × width × depth of color) was used, and compared with the square root of the second parameter (area only). In order to exemplify the cube-root calculations, Table II presents details for one of the groups—in this case the control group of Expt. 1. Similar calculations were then performed for all groups, and are arranged in Table III, along with the results of square-root calculations.

Such simple results are not all, however, that can be learned from data. We found excellent agreement between observers. Correlations of scores between the two observers

TABLE II. Illustration of the Method of Calculating the Values for Length × Width × Color (LWC) shown in Table III.[a]

Exp. 1 (late dosage) control rabbits only. Late (24 hr) controls.

Observer: CGVA				Observer: RPC			
Rabbit	Spot	LWC	$(LWC)^{1/3}$	Rabbit	Spot	LWC	$(LWC)^{1/3}$
1	1	5200	17.32	1	1	6400	18.57
	2	1935	12.46		2	1975	12.54
	3	1874	12.33		3	2800	14.11
	4	1730	12.00		4	1848	12.27
2	1	2825	14.13	2	1	4092	15.99
	2	2493	13.56		2	4921	17.01
	3	894	9.63		3	1463	11.35
	4	2100	12.81		4	2240	13.08
3	1	3570	15.28	3	1	3600	15.33
	2	4380	16.36		2	5376	17.52
	3	2112	12.83		3	2488	13.55
	4	1600	11.70		4	2720	13.96
4	1	786	9.23	4	1	1056	10.18
	2	192	5.77		2	378	7.23
	3	0	0		3	50	3.68
	4	0	0		4	180	5.65
5	1	630	8.57	5	1	750	9.09
	2	520	8.04		2	780	9.21
	3	512	8.00		3	630	8.57
	4	560	8.24		4	770	9.17
6	1	1500	11.45	6	1	1950	12.49
	2	713	8.93		2	960	9.86
	3	820	9.36		3	875	9.56
	4	612	8.49		4	704	8.90
Totals			246.49				278.87

[a] Av $(LWC)^{1/3} = (246.49 + 278.87)/48 = 10.945$; inverse = typical value $= (10.945)^3 = 1310$.

TABLE III. Early Versus Late Dosage of Indomethacin in Schwartzman Reaction; Comparison of Methods of Data.

Type of exp.	Exp. no.	Treatment	No. of rabbits	Cube of mean cube root: Length × width × color (mean)	% Difference	Square of mean square root: Length × width (mean)	% Difference
Early	2	Control	4	586		409	
		Treated	6	10	98	7	98
	4	Control	5	193		121	
		Treated	6	12	94	10	92
	6	Control	6	66		58	
		Treated	6	0	100	0	100
		Overall mean %			97		97
Late	1	Control	6	1310		568	
		Treated	6	596	55	370	35
	3	Control	6	47		47	
		Treated	6	43	9	36	23
	5	Control	6	431		161	
		Treated	6	291	32	147	9
		Overall mean %			32		22

were calculated for all six experiments and were consistent. One early and one late experiment were selected at random for detailed analysis; correlation coefficients were 0.972 for the 44 points of Expt. 4 and 0.970 for the 48 points of Expt. 1. Even if one omits the spots counted as zero, the coefficients, then 0.857 and 0.957, respectively, are highly significant ($p<0.001$). The variability from one rabbit to another was much greater than the variability from spot to spot within a single rabbit. This fact may be seen by the analyses of variance in Tables IV and V.

The magnitudes of the mean squares indicate how much variation can be expected in this kind of work, and may assist others in planning Shwartzman experiments so as to obtain quantitative results.

The overall analysis of variance (approx, based upon mean values from each experiment, taken from Table I) is as shown in Table VI.

TABLE IV. Analysis of Variance, Exp. 1, Observer CGVA.

	df	SS	MS	*F*
Among treatments	1	19.89	19.89	
Among rabbits within treatment	10	1027.17	102.72	22.5[a]
Among spots (within rabbits and treatment)	36	164.56	4.57	

[a] Significantly larger than among spots ($p < 0.001$).

TABLE V. Analysis of Variance, Exp. 4, Observer RPC.

	df	SS	MS	*F*
Among treatments	1	146.40	146.40	
Among rabbits within treatment	9	223.87	24.87	7.17[a]
Among spots (within rabbits and treatment)	33	114.48	3.47	

[a] Significantly larger than among spots ($p < 0.001$).

TABLE VI. Overall Analysis of Variance.

Source of variation	*df*	SS	MS	*F*
Among groups	11	862		
Early versus late	1	226.7	226.7	
Treatment versus control	1	175.5	175.5	
Interaction, above 2 factors	1	88.9	88.9	4.81[a]
Remainder	8	370.9	46.4	
Within groups	56		18.5	

[a] Significant, $p < 0.05$.

Additional calculation: Control versus early treatment: SS = MS = 257.1; $F = 257.1/18.5 = 13.9$ ($p < 0.001$). Control versus late treatment: SS = MS = 7.3; F (7.3/18.5) < 1, not significant.

We see that (i) the overall difference between treatment and control was not significant for the "late" experiments; (ii) the difference between treatment and control was highly significant ($p < 0.001$) for the "early" experiments; and (iii) the overall reduction in the treatment groups relative to control was significantly greater for the "early" experiments than for the "late" ones ($p < 0.05$).

The experiment for tissue studies had results as follows. The six rabbits injected with endotoxin only had histologic scores of 9, 4, 5, 8, 7, and 4. The six rabbits treated with indomethacin before the endotoxin had scores of 10, 12, 9, 11, 11, and 8. The two groups were significantly different ($t = 3.78$), with the probability of chance occurrences of such a difference being less than 0.005.

Discussion. The variability of the Shwartzman phenomenon is such that every feasible means must be taken to reduce it in order to make it a useful quantitative procedure. We believe that Halpern's system (3) of measuring and grading the lesions is valid, and furthermore that by multiplying the values as described here in the methods section, one obtains values readily comparable between laboratories. Halpern's mean control value per rabbit when so calculated proves to be 3958 ($n = 7$) which compares well enough with our own of 3735 ($n = 32$) based on the 24-hr values. Halpern used only one spot per rabbit; the time of the reading was not stated but a personal communication established that it was 24 hr. With our method of injecting four spots per rabbit we have seen some variation within the same animal; an interesting question was therefore whether one would obtain the same total score with four injections as with only one, in the same experiment. The very small value of the mean square among spots, shown in Tables IV and V, shows that little is to be gained by using four spots rather than one. It was surprising to find that in this series of experiments, at least, one could arrive at the same conclusions about effectiveness of drug treatment by merely measuring the area of the lesion, ignoring the depth of color.

Several workers have shown that the Shwartzman and Arthus reactions can be prevented by agents causing neutrophilic leukopenia (4, 5). Thomas (2), confirmed by Halpern (3), showed that the Shwartzman reaction could be duplicated nicely by injection of granules from polymorphonuclear leukocytes. Phelps and McCarty (1) have recently demonstrated that indomethacin inhibits movement of these cells *in vitro*, and that cells from one dog dosed with indomethacin did not migrate normally *in vitro*. We now see that indomethacin can prevent the Shwartzman reaction if it is given before the preparatory injection, but not very well, or perhaps not at all, if it is not given until 1 hr before the eliciting injection.

Nevertheless, indomethacin did not prevent the early infiltration of leukocytes into the injected area. Quite the opposite occurred, in that there were *more* cells in the area in those animals receiving indomethacin. Despite this fact, the lesions usually consequent to the eliciting endotoxin injection were inhibited.

The picture of the local Shwartzman reaction now appears as follows: (i) Neutrophils concentrate in the injected area, as directly seen in this work, and as several workers have already inferred from indirect evidence. (ii) At the time of the eliciting injection, certain enzymic activities are released from these cells; Thomas (2) and Halpern (3) have given evidence that lysosomal granules can duplicate the reaction. We now see that many neutrophils survive the process of degranulation. (iii) If indomethacin is given before the preparatory injection so that it has time enough to have its effect on the granulocytes before they begin assembling at the future

site of conflict, it will prevent the reaction; but if it is not given until the cells have already gathered, it cannot be effective. One infers that indomethacin does not hinder cell motility and does not hinder enzymic processes that occur after release of lysosomal granules, but that it may prevent release of granules from the cell or may prevent the formation of vacuoles in which phagocytized material may be digested.

Summary. Indomethacin inhibited the appearance of the local Shwartzman reaction in rabbits, when given before both the preparatory and the eliciting injections; treated animals at 24 hr had only 8% of the reaction that control animals had. In contrast, when indomethacin was not given until 1 hr before the eliciting injection, the reaction was hindered only slightly; the mean score at 24 hr was 76% of that for controls. Histologic studies have shown that in the presence of indomethacin even more leukocytes appear at the site of endotoxin injection than in control rabbits.

These results can be interpreted as indicating that although indomethacin does not prevent leukocytes from gathering at the site of inflammation, it prevents their disruption and release of enzymes when the second (eliciting) injection of endotoxin is given.

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