ENHANCED RESPONSE OF AN " ACUTE PHASE " SERUM PROTEIN TO REPEATED TISSUE DAMAGE IN THE RAT

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An α_1-glycoprotein of rat serum has been described which increases sharply in concentration following necrosis, tumour growth, infection, turpentine injection and other forms of tissue damage (Darcy, 1964, 1965). This protein is produced in the liver (Neuhaus and Liu, 1964 ; Darcy, 1965 ; Weimer, Benjamin and Darcy, 1966) and there is evidence that its sudden increase in the serum following injury is the result of immediate liver synthesis rather than of a liberation of preformed protein (Chandler and Neuhaus, 1964 ; Neuhaus, 1964). The level of the protein in the serum of female rats is normally about twice as high as in males (Darcy, 1960). During the course of a study of responses to subcutaneous turpentine injection the observation was made that a second injection of turpentine a month after the first evoked a much greater response of the protein. This observation is the subject of the present report. As far as is known the protein has no immunological functions.

MATERIALS AND METHODS

Animals.—Rats of the Chester Beatty (CB) stock were used in some experiments and in others specific pathogen-free rats of the inbred hooded line. The animals were supplied rat cake and water *ad libitum* during the experiments. Blood samples were taken from the tip of the tail except the final bleed of each experiment which was by heart puncture under ether anaesthesia.

Protein determinations.—The specific α_1-globulin was determined by an immunological method (Darcy, 1961). This method gave an error of ± 6 per cent. (95 per cent. confidence limits) for each titration in the present experiments ; the concentration is expressed in units per ml. of serum. Total serum protein was determined by the method of Phillips, van Slyke, Dole, Emerson, Hamilton and Archibald (1958).

Oil of turpentine (commercial) as obtained from hospital dispensaries was employed and injected subcutaneously in one depot of 0·4 ml. per rat. Two batches were employed during the experiments. A bacteriological check run on the 2nd batch showed it to be sterile. Talc powder (French chalk) was obtained from Hopkin and Williams Ltd. It was autoclaved before use and a suspension made in pyrogen-free saline containing 100 mg. of talc per ml.

For experiments in which different groups of animals were employed the groups were selected by means of random numbers from a single batch of the same age. Student's t-test was used to test the significance of differences between means.

RESULTS

First observation.—In the first experiment in which the phenomenon was observed 10 CB male rats 7 weeks of age and mean weight 201 g. were injected with the standard amount of turpentine (0·4 ml.) s.c. in the back. Blood samples

were taken at 0 hr. (just before turpentine) and at 24 and 48 hr. After 35 days the rats (now weighing 439·4 g. on the average) were again given an injection of 0·4 ml. turpentine, but more caudally so as not to overlap the first injection site. The results are shown in Table I. It will be observed that the protein had not returned to the original level by the time of the 2nd injection. Nevertheless the mean increment in the 24 hr. following the 2nd injection was more than double that following the first injection. In spite of the large amount of variability a paired *t*-rest showed that this difference was very highly significant. Likewise the increment from 24–48 hr. was significantly greater after the 2nd turpentine injection. The turpentine lesions removed 2 days after the 2nd injection were examined histologically. They were characterized by a thick wall around the turpentine composed of oedematous tissue, fibrin and densely packed invading cells (polymorphonuclear leucocytes and lymphocytes predominantly) many of which were dead.

TABLE I.—*Response of the Specific Serum α_1-Globulin Following Turpentine Injection into 10 CB Male Rats*

Day	1st injection	2nd injection	*P*
0	0·31±0·87	0·61±0·68	..
1	0·93±0·37	2·05±0·87	..
2	1·81±0·94	3·43±0·99	..
0–1	0·62±0·39	1·44±0·37	<0·001
1–2	0·88±0·59	1·38±0·52	0·02

Mean values (units/ml.) ± standard deviations.

Confirmation with talc.—In the 2nd experiment the question was investigated whether the enhanced second response was specific for turpentine. Twenty specific pathogen-free male rats of the hooded strain were used ; they were 14–16 weeks old and their average weight was 280·5 g. Ten of the rats, selected by means of random numbers, received 0·4 ml. of turpentine s.c. on the left side. After 28 days all the rats were weighed, tail bled, and injected with 100 mg. of sterile talc (in 2 ml. of saline) s.c. on the right side. In the interval the turpentine-injected rats had gained an average of 13·0 g. in weight and the non-injected rats 27·5 g. The rats were tail-bled 24 hr. after receiving the talc and were bled out from the heart under ether at 48 hr. One rat in each group was found to have liver lesions and was discarded. In addition one 48-hr. serum from each group was lost through an accident. The results are shown in Table II. The increment in the turpentine-treated rats was about twice as great as in the controls in the first 24 hr. The total serum protein at 48 hr. in the turpentine-injected rats (6·53 g./100 ml. ± S.D. 0·18 g.) was not significantly different from that in the controls (6·40±0·15), $P = 0{\cdot}1$ It is of interest that the liver (wet weight as percentage of total body weight) was fractionally heavier (3·435 per cent) in the turpentine-injected rats than in the controls (3·259 per cent) although this just failed to reach the 5 per cent significance level ($t_{16} = 2{\cdot}06$, $P \simeq 1$ in 19). " Blindfold " histological examination revealed no difference between the livers or the talc lesions of the 2 groups. No mitoses were observed in the liver sections. The 48 hr. talc lesions were characterized by an invasion of the talc deposit by polymorphonuclear leucocytes (predominantly) and mononuclears and by oedema of the surrounding tissue. Many of the polymorphs were dead.

TABLE II.—*Response of the Specific Serum Protein Following Talc Injection into Male Hooded Rats With or Without Turpentine Injection a Month Previously*

Day	Normal rats	Turpentine-injected rats	*P*
0	0·29±0·03 (9)	0·51±0·14 (9)	..
1	0·86±0·10 (9)	1·60±0·34 (9)	..
2	1·10±0·24 (8)	1·81±0·23 (8)	..
0–1	0·57±0·11 (9)	1·09±0·23 (9)	<0·001
1–2	0·23±0·16 (8)	0·29±0·19 (8)	0·6

Mean values (units/ml.) ± standard deviations. Number of rats in parentheses.

Repeated turpentine injections.—The effect of repeated monthly injections of turpentine was tested on 5 CB male rats 7 weeks old. The day 0 bleed was made before the injection in each instance. The day 2 bleed was omitted for the 3rd injection, but a day 5 bleed was made after each injection. The results are shown in Table III. The 2nd injection of turpentine appears to have again provoked an enhanced response (although the results were not significantly different because of the variability and small number of rats). The 3rd injection did not give an enhanced response. The mean weights at the times of turpentine injection were 256 g., 477 g., 666 g. As the turpentine used for the 3rd injection was from a new batch (the old batch having developed a sediment) the possibility arose that the lower responses to this injection may have resulted from a less toxic turpentine. Accordingly the experiment was repeated on another 5 CB male rats using the fresh turpentine. The results are shown in the lower part of Table III. No enhanced response was observed, and in contrast to the first experiment there was a decline in the level of the specific globulin in the blood between the 2nd and

TABLE III.—*Response of the Specific Protein in the Serum of 5 CB Male Rats Receiving Monthly Injections of Turpentine*

	Day	1st month	2nd month	3rd month
	0	0·33±0·09	0·57±0·28	0·60±0·59
	1	1·05±0·23	1·77±0·79	1·49±0·50
	2	2·45±0·29	3·52±1·62	..
1st experiment	5	2·77±0·57	3·99±1·19	2·25±0·70
	0–1	0·72±0·20	1·20±0·73	± 0·89±0·15
	1–2	1·40±0·13	1·75±1·21	..
	2–5	0·32±0·48	0·47±1·09	..
	0	0·35±0·06	0·27±0·06	0·32±0·07
	1	1·10±0·14	1·07±0·33	1·13±0·33
	2	2·05±0·29	2·16±0·61	2·14±0·87
2nd experiment	5	1·50±0·45	1·23±0·36	1·35±0·52
	0–1	0·75±0·17	0·80±0·34	0·81±0·29
	1–2	0·95±0·21	1·09±0·79	1·01±0·64
	2–5	−0·55±0·24	−0·93±0·89	−0·79±0·38

Mean values (units/ml.) ± standard deviations. Two separate experiments.

5th day after turpentine injection. The uniformity of the responses is noteworthy. The mean weights of the rats at the times of the 3 injections were 161 g., 396 g., and 532 g.

TABLE IV.—*Response of the Specific Protein in the Serum of Male Hooded Rats Receiving Monthly Injections of Turpentine*

Day	1st month	2nd month	3rd month	4th month
0	0·23±0·04 (10)	0·61±0·46 (10)	0·57±0·35 (9)	0·69±0·20 (9)
1	1·49±0·16 (10)	2·27±0·49 (10)	2·54±0·34 (9)	2·62±0·43 (9)
2	2·47±0·34 (10)	3·85±0·47 (10)	4·15±0·35 (9)	4·02±0·55 (9)
5	1·86±0·34 (10)	2·61±0·41 (10)	3·58±0·74 (9)	2·59±0·73 (9)
0–1	1·26±0·15	1·66±0·31	1·97±0·30	1·93±0.35
1–2	0·98±0·29	1·59±0·33	1·61±0·22	1·40±0·34
0–2	2·24±0·33	3·24±0·23	3·58±0·38	3·33±0·46
2–5	−0·61±0·31	−1·24±0·45	−0·57±0·74	−1·43−0·41

Mean values (units/ml.) ± standard deviations. Number of rats in parentheses.

It was decided to repeat the experiment with 10 hooded male rats since these grow much less rapidly than the CB rats and hence the ratio of turpentine volume to body weight would not be so greatly reduced between injections. The rats were 15 weeks old and 309 g. average weight at the start of the experiment. At the times of the 2nd and 3rd monthly injections the average weights were 348 g. and 381 g. respectively. The average dose rates of turpentine were therefore 1·3, 1·15 and 1·05 ml./kg. body weight on the 3 occasions. The results are shown in Table IV. At the time of the 2nd turpentine injection the level of the protein was still high. Nevertheless it gave an enhanced response compared with the first occasion: for the day 0–1 increments $t_9 = 5{\cdot}59$, $P = <0{\cdot}001$ and for the day 0–2 increments $t_9 = 9{\cdot}0$. $P < 0{\cdot}001$, using paired *t*-tests. For the 3rd turpentine injection the response was greater than that for the 2nd injection, the 24-hr. increment being 1·97 units/ml. which was not however statistically significant, $t_8 = 1{\cdot}95$, $P > 0{\cdot}05$); the 2 day increment was likewise higher, $t_8 = 1{\cdot}96$, $P > 0{\cdot}05$). One rat accidentally received its 3rd injection i.m. near the spine, became paralyzed, and had to be destroyed. A 4th injection given one month after the 3rd evoked a response which was not signicantly different from that evoked by the 3rd injection except that there was a greater decline in the level of the specific protein from day 2 to day 5 ($P = 0{\cdot}01$). The rats at this time had an average weight of 389 g. so that the mean turpentine dose was 1·03 ml./kg.

In order to see whether there was a relationship between specific protein response to the first injection and the dose of turpentine per kg. body weight (the range was 1·19–1·39 ml.) the correlation coefficient was calculated for the 24 hr. increment. This gave a value for *r* of 0·65 which was significant ($P = 0{\cdot}05$). The calculated regression line indicated that within this range a weight increase of 16 per cent (and hence a corresponding decrease in dose/kg.) caused a decrease of 0·25 units/ml. of the specific protein in the serum (1·39–1·14 units /ml.) or 18 per cent. There was no correlation between turpentine dose and the day 0–2 increment nor with any of the increments after the 2nd and later injections. It may be noted that the hooded rats gave a larger 24-hr. response to the first injection of turpentine than any of the CB groups in spite of receiving a lower dose of turpentine per kg.

Response in female rats.—In the first experiment (Table V) 10 CB female rats $6\frac{1}{2}$ weeks of age and 189 g. average weight at the beginning of the experiment were injected with turpentine of the old batch and this was repeated a month later

TABLE V.—*Response of the Specific Protein in the Serum of Female CB Rats Receiving Monthly Injections of Turpentine*

	Day	1st month	2nd month	3rd month
1st experiment	0	0·42±0·08	0·69±0·16	0·76±0·12
	1	1·28±0·13	1·43±0·37	1·42±0·34
	2	2·50±0·22	2·68±0·40	..
	0–1	0·86±0·14	0·74±0·25	0·66±0·29
	1–2	1·22−0·18	1·25±0·21	..
2nd experiment	0	0·48±0·10	0·67±0·19	0·68±0·22
	1	0·96±0·16	1·37±0·14	1·38±0·31
	2	1·53±0·53	2·44±0·29	2·65±0·57
	5	1·88−0·29	2·11±0·51	2·18±0·74
	0–1	0·48±0·17	0·70±0·16	0·70±0·25
	1–2	0·57±0·50	1·07±0·31	1·27±0·48
	2–5	0·35±0·36	−0·33±0·34	−0·47±0·49

Mean values (units/ml.) ± standard deviations. 10 rats in the first experiment, 6 in the second.

when their average weight was 278 g. A month later, when their average weight was 336 g. they were given a 3rd injection, this time of the new batch of turpentine.

As will be seen from the table the 2nd injection did not evoke an enhanced response. The 24-hr. increment was slightly, but not significantly ($P = 0{\cdot}2$), lower than after the first turpentine injection. The 3rd injection (with the new turpentine) evoked a still lower 24 hr. response which was significantly different from that for the 1st injection ($P = 0{\cdot}05$).

The experiment was repeated using 6 CB female rats 6 weeks of age and the new batch of turpentine. The responses to the first injection were smaller than in the above experiment but there was a clear-cut enhanced response to the 2nd injection: the 24-hr. increment was significantly higher for the second injection than the first ($P = 0{\cdot}02 - 0{\cdot}01$), but not the increment from 1–2 days ($P = 0{\cdot}1$). The increment from day 2–5 was negative after the 2nd injection and was significantly different from the first ($P = 0{\cdot}05$). A 3rd injection evoked a response which was closely similar to the second (there were no significant differences between them) and the increments of which from 0–1, 1–2 and 2–5 days differed significantly from those following the 1st injection ($P = 0{\cdot}01$, $< 0{\cdot}02$ and $< 0{\cdot}01$ respectively). The mean weights of the rats at the times of the 3 injections were 216 g., 293 g. and 349 g.

The experiment was repeated once more with the new turpentine, this time using 5 female rats of the hooded line. These were $9\frac{1}{2}$ weeks old at the start of the experiment, when their mean weight was 161 g. The results are shown in Table VI. These rats gave a much larger 24-hr. response than the CB rats but this could be accounted for by the higher turpentine dose per kg. body weight. The 24-hr. response was almost twice as great after the 2nd injection ($P = 0{\cdot}01$) giving the enormous increase of 2·41 units of the protein per ml. of serum. There was, unfortunately, no day 2 determination available for the 2nd injection. A 3rd turpentine injection given in the 5th month (when the mean weight was 216 g.) evoked a smaller response than the 2nd ($P = 0{\cdot}05 - 0{\cdot}02$) but larger than the first ($P = 0{\cdot}01 - 0{\cdot}05$ for the 24-hr. increment, $P = 0{\cdot}02 - 0{\cdot}01$ for the 48 hr. increment).

TABLE VI.—*Response of 5 Hooded Female Rats to Monthly Injections of Turpentine*

Day	1st month	2nd month	5th month
0	0·59±0·09	1·11±0·34	0·92±0·11
1	1·87±0·26	3·52±0·22	2·57±0·16
2	2·96±0·60	..	4·03±0·35
5	2·96±0·08	3·30±0·46	4·03±0·63
0–1	1·28±0·24	2·41±0·40	1·65±0·24
1–2	1·09±0·22	..	1·46±0·27
2–5	0·00±0·72	..	0·00±0·30

Mean values of specific protein in serum (units/ml.) ± standard deviations.

Effect of age of rats.—Although the talc experiment (Table II) had shown that the secondary enhanced response was obtained when rats of equal age were used as controls it was decided to repeat this with turpentine. A batch of 16 CB males 7 weeks of age and of mean weight 255·5 g. was divided in two by random numbers and one half was injected with 0·4 ml. of turpentine on the left side. Five weeks later all the rats received 0·4 ml. of turpentine on the right side. At this time the previously injected rats had a mean weight of 490 g. while the other rats weighed 475 g., so that the turpentine dose per kg. body weight was virtually the same in both groups. The specific protein response is given in Table VII. It will be seen that there was no difference in the responses of those rats which had received a previous turpentine injection and those which had not.

TABLE VII.—*Response of the Specific Protein in Male Rats of Equal Age at Time of Receiving a First or Second Injection of Turpentine.*

Day		1st injection	2nd injection	*P*
CB rats	0	0·36±0·29 (8)	0·34±0·29 (8)	0·001
	0–1	0·80±0·18 (8)	0·93±0·18 (8)	0·2
	1–2	1·09±0·28 (8)	0·98±0·47 (8)	0·6
Hooded rats	0	0·24±0·03 (6)	0·48±0·19 (7)	..
	0–1	1·38±0·05 (6)	1·69±0·17 (7)	0·001
	1–1	0·96±0·23 (6)	1·06±0·42 (7)	0·6

Mean serum values (units/ml. ± standard deviations. Number of rats in parentheses.

The experiment was repeated with 13 hooded males 8 weeks old and a mean weight 95 g. at the time that 7 of them received a first turpentine injection. At the final injection 5 weeks later the previously injected rats had a mean weight of 178 g. and the other rats 182 g. The results (Table VII) show a small but highly significant enhanced response at 24 hr. in the rats which were responding to a second turpentine injection.

Haematocrit values, measured by a micro-method, showed changes too trivial to affect the results of either experiment. Turpentine lesions dissected out did not differ significantly in weight between primary and secondary groups in either experiment.

DISCUSSION

A standard amount (0·4 ml.) of turpentine was chosen instead of a dose related to body weight in order to avoid the possible objection that the enhanced response was simply the result of relatively greater tissue damage owing to the larger volume of turpentine injected. This precaution probably weighed heavily against the phenomenon especially in CB rats which increased so greatly in weight in the month between the first 2 injections (approximately 100 per cent in males and 40 per cent in females). Evidence for this was the statistically significant positive correlation between dose per kg. body weight and the 24 hr. increment of the specific serum protein shown in the experiment belonging to Table IV. Other evidence was the large response to 2nd and later turpentine injections shown by the hooded rats whose growth rate is much less (Tables IV and VI). A dosage effect therefore is the most likely explanation of the occasional failure of CB rats to show an enhanced response.

The question whether a 3rd and later turpentine injections will evoke higher responses than preceding injections is examined in Table IV where hooded male rats show a bigger (but not statistically significant) response to the 3rd monthly injection than to the 2nd with the dosage effect weighing against them. The same experiment indicated that a ceiling for the response had been reached around the 3rd injection, since a 4th monthly injection failed to evoke a larger response even though the dose of turpentine per kg. body weight was virtually the same as for the 3rd.

In the experiment using powdered talc (Table II) it was seen that an enhanced response can be evoked by this substance in rats which have been " sensitized " with turpentine. This shows that the effect is not substance-specific. It also makes it probable that the effect is mediated by direct physical damage to the tissues at the site of the inoculum rather than by some chemical leaking out from the turpentine and affecting a central mechanism. It does not rule out the possibility that both turpentine and talc cause the liberation of a particular tissue antigen (or other substance) whose liberation on the first occasion sensitizes the animal so that the second liberation of this substance into the tissues evokes an enhanced tissue and protein response. Against this interpretation, however, is the failure to find any macroscopic or microscopic difference between the talc lesions of turpentine-sensitized rats and control rats. The evidence of heavier livers in the sensitized rats probably reflects the greater synthesis of the specific globulin in that organ. A question of major interest is whether the liver itself is " sensitized " in a way analogous to the lymphoid tissue of an immunized animal or whether the stimulus coming to the liver after the 2nd tissue injury is greater—or whether both factors are operating together. The evidence suggests that the local tissue injury is not greater in the sensitized animal. Histologically the chief characteristics of both turpentine and talc lesions are a walling-off of the irritant by oedema, fibrin and numerous polymorph and mononuclear leucocytes. It is probable that the widespread destruction of the invading polymorphs contributes to triggering the response (Darcy, 1964).

An important finding was the large contribution made to the enhanced response by simple ageing of the rats by one month, particularly in CB rats where it appears to account for most of the phenomenon. This underlines the need in future studies of controls for age as well as for dosage. It suggests that the effect of age in the response of rats to trauma is well worth an investigation of its own.

SUMMARY

The level of an α_1-glycoprotein in the blood of rats increased sharply following the subcutaneous injection of 0·4 ml. of turpentine. When the injection was repeated a month later an enhanced response was obtained. It was found possible to obtain a still greater response to a 3rd monthly turpentine injection, but not to a 4th. The occasional failure to obtain an enhanced response could be accounted for by a lower dose of turpentine per kg. body weight.

When talc suspension was injected a month after turpentine an enhanced response was also obtained.

The phenomenon can be partly accounted for by an increased responsiveness of the rats with age, but at least in hooded rats there is a part which cannot be explained.

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REFERENCES

CHANDLER, A. M. AND NEUHAUS, O. W.—(1964) *Am. J. Physiol.*, **206**, 169.

DARCY, D. A.—(1960) *Br. J. Cancer*, **14**, 524.—(1961) *Nature, Lond.*, **191**, 1163.—(1964) *Br. J. exp. Path.*, **45**, 281.—(1965) *Br. J. exp. Path.*, **46**, 155.

NEUHAUS, O. W. AND LIU, A. —(1964) *Proc. Soc. exp. Biol. Med.*, **117**, 244.

PHILLIPS, R. A., VAN SLYKE, D. D., DOLE, V. P., EMERSON, K., HAMILTON, P. B. AND ARCHIBALD, R. M.—(1958) In ' Quantitative Clinical Chemistry ', Vol. 2, Baltimore (Williams and Wilkins), p. 941.

WEIMER, H. E., BENJAMIN, D. C. AND DARCY, D. A.—(1966). *Nature, Lond.*, **208**, 1221.