INFLAMMATORY RESPONSES IN NORMAL AND ADRENALECTOMIZED RATS DURING OBSTRUCTIVE JAUNDICE

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The reversibility of the rheumatoid process observed during hepatitis and jaundice (1) has been generally explained in terms of increased corticosteroidal activity, due either to retention of bile acids, which contain chemical nuclei of a steroidal nature similar to those of adrenal cortical hormones (2), or to the ability of bilirubin to displace corticosteroids from sites of protein binding (3). Little attention has been given to the possibility that different mechanisms are involved in the inhibition of inflammation which takes place during jaundice.

Our interest in this problem was stimulated on reading that in rats ligation of bile duct brings about an inhibition of inflammatory lesions produced by Freund's adjuvant (4). This result led us to investigate the anti-inflammatory effect of jaundice by means of an experimental scheme analogous to that generally employed to evaluate antirheumatic drugs.

METHODS

Experiments were conducted on Long-Evans rats, weighing 120-180 g and kept at 22-23°C. Animals of both sexes were used, but results have been pooled since no significant difference was observed between male and female animals.

Surgical procedures, performed under ether anesthesia, were followed by intraperitoneal injection of 20,000 I.U. of Na penicillin for animal. Bile duct ligation was carried out through an incision in the ventral midline. By raising and extending the duodenal ansa, the mesentery was disclosed and the bile duct located; then a double ligation was made. Adrenalectomy was performed through an incision of the skin in the dorsal midline. Postoperatively, rats were given drinking water containing 0.9% NaCl.

The same surgical procedures were performed in sham operated rats, without ligating the bile duct or removing the adrenals.

Inflammatory responses were studied by injecting carrageenin (0.05 ml of 1% suspension) into the plantar surface of the rat's hind paw (5). The foot volume was measured according to Lenčič (6). The cotton pellet induced granuloma was studied according to Meier *et al.* (7). At the end of experiments animals were autopsied and the weight of

main internal organs was determined. Granulomas were weighed after drying for 24 hours at 60°C. In some experiments the compounds under investigation were dissolved in ethanol (95%) and added to the pellets, which were then dried.

To study heat denaturation, rat serum was diluted 1:1 with a phosphate buffer (M/10; pH 7.4) and heated at 70°C for 2 minutes. The compounds under investigation were dissolved in the phosphate buffer. Denaturation was determined by electrophoresis on cellulose acetate strips (2.5×16 cm), with the use of sodium diethylbarbiturate and diethylbarbituric acid buffer (μ 0.044; pH 8.6) at a voltage of 200 V for 60 minutes. Experiments were also conducted on bovine serum albumin, corresponding to the 5th fraction and containing 3% globulin. According to Mizushima (8) a 1% solution of albumin in a phosphate buffer (M/15; pH 5.3) was diluted 1:1 with water and heated at 70°C for 100 seconds. Denaturation was assessed by turbidimetry, with a spectrophotometer set at 640 m μ . Aqueous solution of the compounds or serum samples under investigation were added to the albumin solution before heating.

RESULTS

Table 1 shows the results obtained in investigating the response to carrageenin of normal animals, sham-operated animals, and animals with ligature of the bile duct.

TABLE 1. Experimental jaundice, produced in rats by ligature of bile duct, and carrageenin edema.

Experimental groups	Time interval from operation	No. of rats	Initial foot volume	Edema in ml at different time intervals from carrageenin injection		
				2 hr	4 hr	6 hr
A Control rats	/	10	0.845	0.788	1.150	1.050
B Sham-operated rats	1 day	10	0.821	0.592 ¹⁾	0.864 ¹⁾	0.783 ¹⁾
C Operated rats	1 day	10	0.811	0.339 ²⁾	0.619 ²⁾	0.574 ²⁾
D Control rats	/	10	0.881	0.761	0.903	0.767
E Sham-operated rats	2 days	10	0.875	0.578	0.842	0.721
F Operated rats	2 days	10	0.850	0.191 ³⁾	0.419 ³⁾	0.300 ³⁾
G Control rats	/	15	0.850	0.543	0.743	0.706
H Sham-operated rats	3 days	10	0.745	0.621	0.757	0.683
I Operated rats	3 days	14	0.701	0.288 ⁴⁾	0.436 ⁵⁾	0.395 ⁶⁾

The following comparisons among various groups of animals revealed significant differences :

- 1) A-B, $P < 0.01$
- 2) A-C, $P < 0.001$; B-C $P < 0.05$
- 3) D-F, $P < 0.001$; E-F $P < 0.001$
- 4) G-I, $P < 0.01$; H-I $P < 0.001$
- 5) G-I, $P < 0.01$; H-I $P < 0.01$
- 6) G-I $P < 0.001$; H-I $P < 0.01$

The response to carrageenin was studied, in different groups of animals, one, two, and three days after the surgical procedure. In sham-operated rats carrageenin edema was less marked than that in normal controls only on the first day subsequent to the surgery. Ligature of the bile duct inhibited the inflammatory response to carrageenin. This effect is statistically significant on all three days following the surgery when compared both with the normal controls and with the sham-operated animals.

These data suggested that an interval of three days between bile duct ligature and assessment of inflammatory response could be considered sufficient to eliminate the non-specific influences of the surgical procedure.

Table 2 shows the results from a study of carrageenin edema in rats that were adrenalectomized and subjected to bile duct ligature.

It can be noted that ligature of the bile duct causes a clearcut inhibition of carrageenin edema also in the adrenalectomized rat. There exists instead no significant difference between adrenalectomized animals and sham-operated controls.

A study of cotton pellet induced granuloma was carried out in trials of three days, so as to prevent a prolonged jaundice from producing severe cachectic phenomena. The data reported in Table 3 demonstrate that an experiment of this duration is sufficient

TABLE 2. Experimental jaundice in adrenalectomized rats and carrageenin edema. Adrenalectomy and ligature of bile duct were performed respectively 3 and 4 days before the experiment.

Experimental groups	No. of rats	Initial foot volume	Edema in ml at different time intervals from carrageenin injection		
			2 hr	4 hr	6 hr
A					
Adrenalectomy	10	0.939	0.540	0.728	0.702
B					
Sham adrenalectomy	9	0.895	0.642	0.822	0.692
C					
Adrenalectomy+ Bile duct ligature	9	0.915	0.191 ¹⁾	0.290 ¹⁾	0.297 ¹⁾

The following comparisons among various groups of animals revealed significant differences :

1) A-C, $P < 0.001$; B-C, $P < 0.001$

TABLE 3. Effects of hydrocortisone acetate on cotton-pellet granuloma and on thymus, spleen and adrenal weights.

The compound was administered for 3 days at a daily dose of 20 mg/kg s.c.

Groups	No. of rats	Body weight g		Granuloma dry weight mg	% Body weight			
		Beginning	End		Granuloma	Thymus	Spleen	Adrenals
Methylcellulose	14	124.3	138.7	122.9	0.090	0.168	0.373	0.024
Hydrocortisone	15	120.1	118.9	56.6 ¹⁾	0.046 ¹⁾	0.042 ¹⁾	0.286 ²⁾	0.021

The following comparisons between the two groups of animals resulted in significant differences :

1) $P < 0.001$, 2) $P < 0.01$

to disclose hydrocortisone's activity.

Hydrocortisone, administered daily at a dose of 20 mg/kg s.c. for three days, inhibited body weight increase and suppressed weight of granuloma, thymus, and spleen. Effects on the adrenals, though significant when the absolute weight of these organs is considered (average in control and treated group: 33.5 and 35.9; $P < 0.001$), are not significant when the ratio of adrenals to body weight is employed for comparison.

Table 4 shows the results obtained in an investigation of the influence of bile duct ligation on granuloma, body weight, and weight of thymus, spleen, and adrenals.

In this case, too, the trials were carried out over a period of three days. No significant difference was noted between normal control animals and sham-operated animals. Ligation of the bile duct caused a significant inhibition in the weight of the thymus. Granuloma proved to be inhibited in an absolute sense, whereas in terms of its ratio to body weight no significant difference from the control groups was observed. The weight of the spleen

TABLE 4. Experimental jaundice in rats and cotton-pellet granuloma, thymus, spleen and adrenal weights.

Ligation of bile duct was performed 3 days before autopsy.

Experimental groups	No. of rats	Body weight		Granuloma dry weight mg	% Body weight			
		Beginning	End		Granuloma	Thymus	Spleen	Adrenals
A Control rats	21	163.9	169.8	156.2	0.096	0.128	0.329	0.023
B Sham-operated rats	20	154.4	155.0	158.5	0.101	0.137	0.381	0.028
C Operated rats	35	163.1	145.4	129.6 ¹⁾	0.090	0.080 ²⁾	0.315 ³⁾	0.027

The following comparisons between groups of animals revealed significant differences :

- 1) B-C, $P < 0.02$
- 2) B-C, $P < 0.001$
- 3) B-C $P < 0.05$

TABLE 5. Experimental jaundice in adrenalectomized rats and cotton-pellet granuloma, thymus and spleen weights.

Adrenalectomy and ligation of bile duct were performed, respectively, 3 days and 4 days before autopsy.

Experimental groups	No. of rats	Body weight		Granuloma dry weight mg	% Body weight			
		Beginning	End		Granuloma	Thymus	Spleen	Adrenals
A Adrenalectomy	14	175.5	169.0	116.9	0.069	0.162	0.428	/
B Sham adrenalectomy	10	154.3	160.0	112.7	0.070	0.135	0.337	0.025
C Sham adrenalectomy Bile duct ligation	25	171.0	156.0	103.3	0.065	0.084 ¹⁾	0.345	0.027
D Adrenalectomy Bile duct ligation	20	177.5	170.0	112.7	0.066	0.123	0.374	/

Comparisons among various groups of animals revealed the following significant differences :

- 1) B-C, $P < 0.01$; C-D, $P < 0.02$

showed, following bile duct ligation, a significant reduction when compared with the sham-operated controls, but not with the normal controls.

The effects of bile duct ligation on cotton pellet granuloma, thymus, and spleen were investigated also in adrenalectomized animals. The animals were examined four days after adrenalectomy and three days after bile duct ligation. The results obtained are reported in Table 5.

In the adrenalectomized animals ligation of the bile duct produced no significant effect.

Another group of trials was carried out to investigate any possible anti-inflammatory contact effect of some of the substances whose blood level increases during jaundice. Bilirubin, dehydrocholic acid, deoxycholic acid, and cholic acid (the latter one as the sodium salt) were added to the cotton pellet in doses up to 0.5 mg without obtaining any effect. It should be noted that in the same test hydrocortisone is active at a dose of 0.1 mg. Bilirubin also submitted to the carrageenin test being injected in suspension together with carrageenin. At doses up to 125 μ g no effect was obtained, whereas hydrocortisone was active at a dose as low as 5 μ g.

The results obtained in trials carried out to investigate the response to heat of serum of normal rats, rats submitted to bile duct ligation, and sham-operated rats are shown in Table 6.

The serum of jaundiced animals proved to be more resistant to heat than that of the controls. This phenomenon, already evident 24 hours after ligation of the bile duct, was further accentuated 72 hours afterwards, when there occurred also a decrease in the albumin

TABLE 6. Response to heating (70°C for 2 minutes) of rat serum during jaundice.

Denaturation was studied by electrophoresis and is expressed in terms of residual albumin.

Time interval from operation	Material	No. of rats	% of albumin		Residual albumin in %
			Before heating	After heating	
24 hr	Jaundiced rats	9	55	19.6	35.7
	Sham-operated rats	5	59.2	5	8.45
72 hr	Jaundiced rats	10	45	24	53.4
	Sham-operated rats	5	56.6	3	5.4
	Normal rats	10	54	3	5.4

TABLE 7. Effects of serum from jaundiced rats on heat denaturation of bovine serum albumin.

Serum dilutions and albumin solutions were added 1 : 1.

The average of 5 determinations is reported.

Time interval from operation	Serum dilutions	Turbidity of albumin added with serum from		
		Normal rats	Sham-operated rats	Jaundiced rats
24 hr	1 : 10	1560	1390	834
	1 : 50	1650	1650	1650
72 hr	1 : 10	1400	1590	700
	1 : 50	1590	1675	1547

level. The experiment, repeated in adrenalectomized animals, showed also in this case an increased resistance to heat of the serum after bile duct ligation.

A last experiment was conducted to establish whether the serum of the jaundiced rat was capable of imposing an increased resistance to heat also on a different and surely normal protein material. The serum of jaundiced animals was diluted and added to a solution of bovine serum albumin. The results obtained from a study of the response to heat of the latter material are shown in Table 7.

It can be noted that the serum of the control animals, both normal and those sham-operated, up to a 1:10 dilution, imposes on the albumin solution a greater resistance to heat, which is moderate in extent but constant. The serum of the jaundiced animals explicates the same effect, though to an extremely more marked extent. It can also be noted that the anti-denaturant action of jaundiced serum is more evident after 72 hours than after 24 hours following bile duct ligation. With 1:50 diluted serum no significant effect on the turbidimetric response of albumin to heat was observed.

To establish whether the anti-denaturant action of serum is influenced by the adrenals, the glands were excised from four rats, which on the following day were submitted to ligation of the bile duct. Allowing 72 hours to pass after the second surgical procedure, the serum was collected, diluted 1:10, and placed in contact with a solution of bovine serum albumin. The response to heat of this mixture proved to be inhibited by 45%. This suggests that the adrenals play a role of little importance in these phenomena. These results support the hypothesis that the substances accumulating in the serum during jaundice are endowed with anti-denaturant activity. It was hence thought desirable to investigate the action of certain bile salts and bilirubin on heat denaturation of bovine serum albumin and rats serum. The results obtained are shown in Table 8.

All the bile salts examined inhibited denaturation of bovine serum albumin, whereas on rat serum only sodium deoxycholate explicated an evident action. Bilirubin inhibited denaturation of rat serum, but not of bovine serum albumin. Its lack of effect on this latter protein material seems imputable to its insolubility at the pH employed in this second trial (9). Another trial was carried out by diluting rat serum 1:1 with a solution containing sodium cholate and bilirubin, each at a concentration of 200 $\mu\text{g/ml}$. The serum's response to heat proved to be inhibited by 31%.

TABLE 8. Effects of some biliary salts and bilirubin on the heat denaturation of bovine serum albumin (B.S.A.) and rat's serum.

		Inhibition % of denaturation at the following concentrations ($\mu\text{g/ml}$)			
		300	200	100	50
Sodium cholate	B.S.A.	29	20	13	7
	Rat serum	4.74	4.74	0	—
Sodium deoxycholate	B.S.A.	27	26	17	10
	Rat serum	33.2	9.5	2.37	0
Bilirubin	B.S.A.	0	0	—	—
	Rat serum	24	18	10	7

A last investigation was conducted to establish whether adrenalectomy could influence the sensitivity of serum to bilirubin's anti-denaturant action. The serum of rats adrenalectomized four days before was diluted 1:1 with a solution containing 300 $\mu\text{g/ml}$ of bilirubin. The response to heat proved to be inhibited by 20%, a value similar to that obtained with normal rat serum.

DISCUSSION

Table 9 illustrates schematically the main effects of jaundice in the normal and adrenalectomized rat, comparing them with the effects explicated by two typical antirheumatic drugs.

TABLE 9. Comparative picture of jaundice and antirheumatic drugs.

	Thymus and spleen involution	Granuloma inhibition	Carrageenin edema inhibition	Protein denaturation inhibition
Experimental jaundice in intact rats	+	—	+	+
Experimental jaundice in adrenalectomized rats	—	—	+	+
Hydrocortisone	+	+	+	—
Phenylbutazone	—	+	+	+

Let us discuss first of all the thymolytic effect explicated by jaundice, which does not manifests itself in the animal submitted to bile duct ligation 24 hours after adrenalectomy. Since this time interval is sufficient to bring the blood level of corticosterone from 17.6 to 2.67 $\mu\text{g/ml}$ (10), it can be postulated that the involution of the thymus requires the mediation of adrenal hormones. It is furthermore known that the glucocorticoid hormones inhibit the thymus. It could hence be supposed that the bile salts influence in some way adrenal secretion or hormone metabolism, or that bilirubin can displace, as Brodie (3) postulated, the hormones bond to the protein. However, this last interpretation does not explain why phenylbutazone, which like bilirubin competes with corticosterone at the protein bond (11), explicates no thymolytic effect (12).

Our investigations also demonstrated that experimental jaundice in the rat does not involve any inhibition of cotton pellet induced granuloma. This result seems surprising for two reasons. First of all, antirheumatic substances usually inhibit this response. Secondly, in all the corticosteroids employed in antirheumatic therapy a parallelism between effect on the thymus and on granuloma has been noted (12, 13). In this regard, however, it is necessary to consider that corticosterone, the main glucocorticoid in the rat, presents no antirheumatic action in man (14). Admitting then that jaundice activates in man and in the rat a response of the corticoadrenal type, we might be led to postulate a difference in the anti-inflammatory effects of jaundice on the basis of the diverse physiological properties of the glucocorticoid hormones present in the two species. Apparently this hypothesis clashes with the observation that ligation of the bile duct inhibits in the rat arthritis

induced by Freund's adjuvant, which thus far has shown a pharmacological sensitivity very similar to that of the rheumatic diseases and arthritis in man. To arrive at a definitive clarification of this point, it would be necessary to investigate the effects of corticosterone on arthritis induced by Freund's adjuvant. There exists, however, another possibility, that is, that the corticoadrenal response is responsible only partially for the anti-inflammatory effects of jaundice.

This hypothesis seems, first of all, to have found confirmation in the results of investigations of carrageenin edema, which is inhibited by jaundice also in adrenalectomized animal. Since this is a response considered typically inflammatory, the results seem to demonstrate that jaundice can inhibit inflammation also with a mechanism entirely independent of adrenal function. It is, however, necessary to recall that the edema produced by irritating agents can also be inhibited by hypothermia (15), and that hence the anti-edema action of jaundice could be related to the phenomena of denutrition and hypothermia present in the jaundiced rat. More pertinent, in this regard, is the observation that in the serum of the jaundiced rat, whether normal or adrenalectomized, there accumulate substances capable of inhibiting protein denaturation. These results, indeed, are related to the researches of Mizushima and cooperators (8, 16, 17), which have subsequently been confirmed in our laboratories (18). These researches tend to explain the action of the nonsteroidal antirheumatic drugs in terms of anti-denaturant action on proteins, and to relate the action of these drugs to a mechanism different from that attributed to the cortisone-like substances. A correlation between anti-denaturant action and anti-inflammatory action still represents a working hypothesis. It should be noted, however, that a further confirmation of such a correlation is provided by a recently described investigation of an anti-inflammatory drug (19, 20).

A further point must be made about bilirubin and the bile salts, to which our trials led us to attribute the anti-denaturant effect of jaundiced serum. These substances are active at concentrations corresponding to those reached, in reality, in man during obstructive jaundice and hepatitis (21). This circumstance provides the basis for stating that in human pathology, too, there must exist alterations of the protein response analogous to those documented in the rat. The possibility, therefore, that jaundice may at least in part act against inflammation with a mechanism of this type appears to merit attentive consideration.

SUMMARY

Three days after bile duct ligation the rat presented involution of the thymus, inhibition of carrageenin edema, and a decrease in the heat denaturation of serum. No inhibition of cotton pellet granuloma was observed. Adrenalectomy prevented effects of jaundice on thymus, but not on carrageenin edema and on the response of serum to heat. Jaundiced serum inhibited, up to a 1:10 dilution, heat denaturation of bovine serum albumin. An antidenaturant effect was detected in bilirubin, sodium cholate, and sodium deoxycholate. These substances have no effect when added to carrageenin or to cotton pellets at concentrations higher than the active ones for hydrocortisone.

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