Alterations of tumor necrosis factor-alpha and interleukin 6 production and activity of the reticuloendothelial system in experimental obstructive jaundice in rats

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Background

Immunological changes are well recognised in obstructive jaundice. The aim of this study was to monitor plasma tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) levels in rats with obstructive jaundice.

Methods

The ability of splenocytes and peritoneal exudate cells (PEC) to produce these cytokines both spontaneously and on induction with lipopolysaccharide (LPS), was compared in rats with and without obstructive jaundice (OJ). The activity of the reticuloendothelial system (RES) was also measured.

Results

Serum cytokine levels in OJ rats were higher than in control rats. PEC cultures produced significantly more IL-6, compared with control rats, declining thereafter. TNF-α activity in the splenocyte cultures of OJ rats was also higher than in the control group. Pronounced differences were found in the ability to produce TNF-α by PEC, i.e., TNF-α production was much stronger on day 7 in OJ rats than in controls. On day 14 TNF-α production was much lower and the spontaneous response was equal to the LPS-induced one. On day 21 the cells of OJ rats partially regained the ability to produce TNF-α. RES activity of OJ rats was significantly suppressed in the liver and spleen, whereas the phagocytic activity in the lungs was elevated.

Conclusion

We have demonstrated that the immune reactivity of OJ rats, intially elevated, underwent subsequent depression. The study also revealed a major effect of the operation alone on the studied parameters.

Keywords

Experimental; obstructive jaundice; TNF-α; IL-6; reticuloendothelial system

Introduction

The pathology of obstructive jaundice (reviewed in [1]) is associated with translocation of bacteria from the gastrointestinal tract to mesenteric lymph nodes [2,3] and stimulation of the reticuloendothelial system cells by endotoxins [4,5]. These events result from impairment of liver function [6–8] and lower concentration of bile acids, the role of which is to inactivate endotoxins [9]. Constant activation of the immune system by endotoxins leads to a state of hyporeactivity both in patients [10] and experimental animal models [3], which creates an abnormal response to operation [12,13].

Together with humoral mechanisms, reticuloendothelial activity constitutes an integral part of the human defensive system [14,15]. Nearly 85–90% of macrophages are collected as the Kupffer cells in the venous sinusoids of the liver and spleen [16,17]. Reactivity of RES cells is reflected by their ability to produce mediators of the immune response such as TNF-α, IL-6, superoxide anion and nitric oxide and to phagocytose various particles, such as bacteria, immunological complexes, fibrinogen degradation products and endotoxins [3,14, 18–22]. Clinical and experimental studies [23,24] have shown that the overall activity of the RES is markedly depressed in

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shock, haemorrhage, postoperative trauma, endotoxaemia and in most hepatic diseases. The main consequence of inhibiting RES function in the liver and spleen is to overcome this protective barrier, which leads to the development of general endotoxaemia [1, 6, 25].

Both cytokine production and phagocytic activity of cells are generally found to be depressed in obstructive jaundice (OJ), [3,8,11,12,14], although stimulation of immune system cells has also been observed [18,19,21]. The immune status of experimentally jaundiced animals has usually been tested on days 7–14 or even on day 21 after the onset of OJ [3,14,18]. Focusing at specific time intervals during the course of OJ was probably the main cause of inconsistent conclusions with regard to the immune status of OJ animals. We therefore wished, to study the immune status of rats during both the early and late phase of experimental OJ. In addition, cytokine production was related to the phagocytic activity of RES cells on day 14. More specifically, the aim of this work was to determine serum levels of TNF-α and IL-6 and the ability of spleen cells and peritoneal exudate cells to produce these cytokines both spontaneously and upon stimulation with LPS on days 7, 14, and 21 of OJ. In addition, selective organ uptake of the colloidal sulfur-labeled Technetium (Tc 99mm) was determined as the indicator of RES activity in the liver, spleen and lung on day 14 of OJ.

Materials and methods

Animals

Male rats of the Buffalo strain (170–270 g, mean 230 g) were used. Rats were maintained in stable conditions and were fed standard commercial diet with free access to food and water.

Surgical procedure

All procedures were performed under light ether anaesthesia in clean but not sterile, conditions as described before [26]. Briefly, the abdominal cavity was opened with a midline incision after skin disinfection. The common bile duct (CBD) was ligated in its supraduodenal portion between the lowermost tributary of the bile duct and the uppermost tributary of the pancreatic duct. Control (sham operated) rats underwent opening of the abdominal cavity and dissection of the CBD without ligation. The abdominal cavity was closed with a continuous two-layer suture, which was removed after 7 days.

Organ uptake of colloidal sulphur-labeled technetium

In the study of the RES organ activity, colloidal sulphur-labelled Technetium (Tc 99 mm) was used (Center of Reactor and Isotope Production, Otwock, Poland). As soon as the rats were anaesthetised, 0.5 ml isotopic tracer was injected through the tail vein (total radioactivity of injected tracer 2–3.5 $\times$ 10^5 impulses per minute), and the organs were isolated 15 minutes later. Radioactivity of the organs was measured by a scintillation counter (SSU-70-2). RES activity was estimated as total and specific organ uptake. The total organ uptake of the liver, spleen and lungs was expressed as a percentage activity of injected tracer. The specific organ uptake was the organ uptake calculated per 1 g of the tissue.

Serum peritoneal and spleen cell cultures were prepared and cytokines were induced in cell cultures, as described in detail elsewhere [27].

Determination of IL-6 activity

The assay was performed according to Van Snick *et al.* [28]. Briefly, 7TD1 indicator cells were washed 3 times with Hanks' medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics at 2 $\times$ 10^4 cells/ml. Then, the cells were distributed in 100 µl aliquots into 96-well flat-bottom plates containing 100 µl of serially diluted plasma or supernatant in triplicate. After 72 hours of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method [29]. The results of IL-6 activity are presented in pg/ml; such concentration of IL-6 corresponds to the activity of IL-6 expressed in units/ml [28]. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample in which a half-maximal proliferation of 7TD1 cells was registered. The sensitivity limit in this assay is 0.5 pg IL-6 when tested with recombinant IL-6. The 7TD1 line responds by proliferation only to IL-6 [28].

Determination of TNF-α activity [30]

For determination of TNF-α activity a highly specific indicator clone WEHI 164.13 was used. The cells were washed three times with Hanks' solution and resuspended in RPMI 1640, supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2 $\times$ 10^5/ml. Cells were then distributed into 96-well, flat-bottom plates (2 $\times$ 10^4/well). Serially diluted plasma samples were prepared on separate

plates and were transferred to microtitre plates containing WEHI 164.13 cells. The medium contained in addition 1 μg/ml actinomycin D to increase sensitivity of the assay. After overnight incubation, the survival of cells was determined using an MTT colorimetric assay [29]. The results of TNF-α activity are presented in pg/ml, where 10 picograms of TNF-α correspond to 1 unit of activity when tested with a recombinant human TNF-α. The sensitivity limit of this assay is 2.5 pg TNF-α.

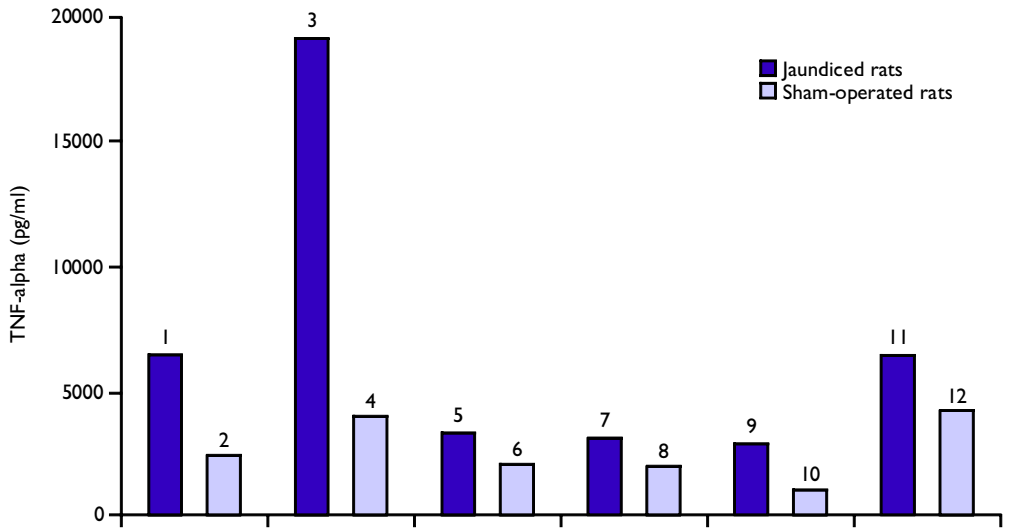
Statistics

Results are presented as mean values from several rats (as indicated in figure legends ± standard deviation). In the statistical evaluation of the results with regard to TNF-α and IL-6, one way analysis of variance for completely randomised block design for 3 levels (influences of the studied subgroups of rats, presence of OJ and its persistence) was applied. In other studies Student's t-test, Cox-Cochran's test and paired data were applied. The differences were regarded as significant when $p \leq 0.05$.

Results

The ability to produce TNF-α by splenocytes and peritoneal exudate cells in the course of OJ

Preliminary studies showed that the ability of peritoneal exudate cells to produce LPS-induced TNF-α gradually increased when measured on days 2, 3 and 7 after onset of OJ, but then tended to decline on day 14 (data not presented). Figure 1 shows changes in the ability of PEC to produce TNF-α both spontaneously and upon LPS stimulation on days 7, 14 and 21 of OJ and sham-operated rats. On day 7 both spontaneous and LPS-induced TNF-α values in OJ rats exceeded control values by several fold.



	1 week				2 week				3 week			
	Spontaneous		LPS induced		Spontaneous		LPS induced		Spontaneous		LPS induced	
No of rats	15	15	15	15	9	10	9	10	19	10	19	10
Mean Value +/−	6.433	2.489	19.126	4.105	3.310	2.105	3.188	2.102	3.099	1.218	6.495	4.395
SD	4.494	1.946	19.513	2.691	1.892	1.427	1.989	872	2.981	682	3.237	2.243

Statistical difference: 1:5 (p=0.0470), 1:9 (p=0.035), 3:7 (p=0.01), 3:11 (p=0.034), 4:8 (p=0.03), 8:12 (p=0.026), 1:2 (p=0.004), 3:4 (p=0.006), 9:10 (p=0.013), 11:12 (p=0.002), 1:3 (p=0.01), 2:4 (p=0.02), 9:11 (p=0.028), and 10:12 (p=0.001), 2:10 (near statistical difference), other relations not statistically significant.

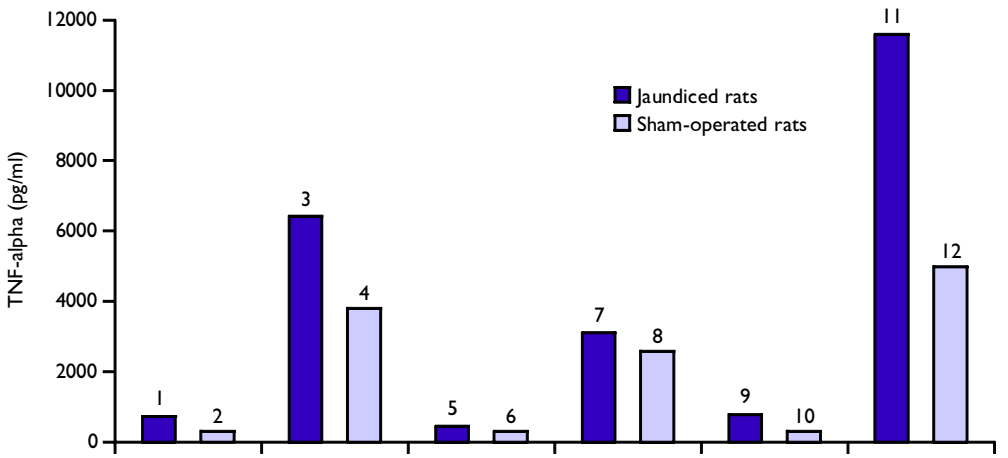
Figure 1. *Profile of TNF-alpha activity in peritoneal exudate cell cultures of rats with (1–3)-week obstructive jaundice and in sham-operated controls.*

The production of TNF-α in both groups of rats changed dramatically on day 14. Inhibition was more profound in the case of OJ rats, but the most characteristic feature was a lack of difference between spontaneous and LPS-induced cytokine production. That phenomenon disappeared on day 21, although the ability to produce TNF-α remained low. On that day the inducibility of TNF-α by LPS in control rats was higher than that of OJ rats. Both spontaneous and LPS-induced TNF-α production on day 21 were, however, much higher in experimental rats than controls, (129 versus 707 pg/ml).

A different pattern of changes in the production of TNF-α was exhibited by splenocyte cultures (Figure 2). In this system the spontaneous cytokine production was low in both groups, and much higher TNF-α levels could be induced by LPS at all times. In general, high TNF-α values induced on day 7 declined on day 14 and rose significantly on day 21 to exceed the inducible TNF-α levels registered on day 7. In addition, the differences in inducible TNF-α concentration between the studied groups were less pronounced on day 14. Control levels of spontaneous and LPS-induced TNF-α on day 21 in control, untreated rats were much lower (76 and 149/pg/ml) than those in operated rats.

The ability to produce IL-6 by splenocytes and PEC cells in the course of OJ

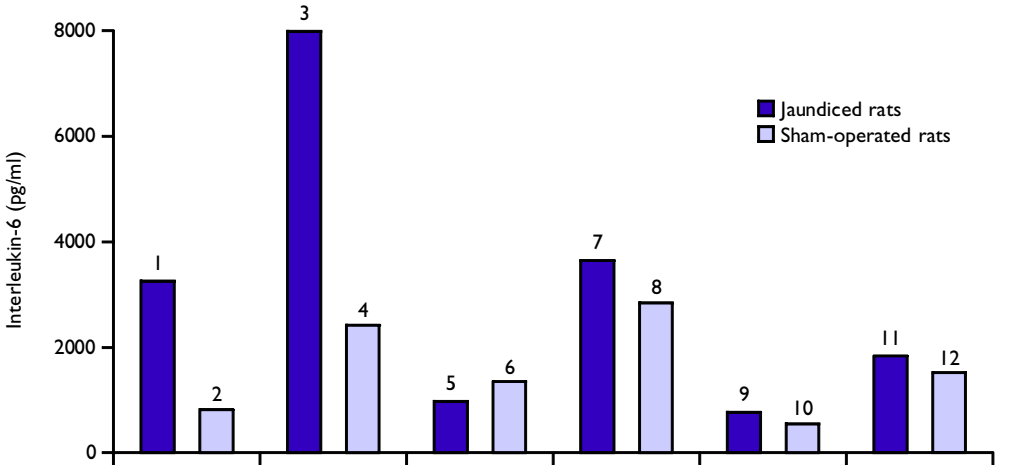
The production of IL-6 by PEC cultures in the course of OJ and in sham-operated rats is shown in Figure 3. Profiles of IL-6 production differed between the studied groups: in OJ a high ability to produce IL-6 on day 7 underwent a gradual but highly sigificant decline (for both types of IL-6 production). In control rats, by contrast, IL-6 production was comparable on days 7 and 14 and lower on day 21, but these differences did not reach statistical significance. IL-6 production in PEC cultures from control rats was low: 175 and 449 pg/ml for spontaneous and LPS-induced IL-6.



	1 week				2 week				3 week			
	Spontaneous		LPS induced		Spontaneous		LPS induced		Spontaneous		LPS induced	
No of rats	10	10	10	10	20	23	20	23	9	10	9	10
Mean Value +/−	765	316	6.424	3.802	472	332	3.140	2.654	775	310	11.593	4.976
SD	529	181	3.438	2.107	464	179	2054	1.263	474	230	5.366	1.480

Significant differences: 3:7 (p=0.017), 3:11 (p=0.002), 3:7 (p=0.01), 7:11 (p=0.001), 8:12 (p=0.001), 1:2 (p=0.02), 9:10 (p=0.013), 11:12 (p=0.002), 1:3 (p<0.001), 2:4 (p<0.001), 5:7 (p<0.001), 6:8 (p<0.001), 9:11 (p<0.001), and 10:12 (p<0.001), 1:5, 5:9, 4:8 and 3:4 (near statistical difference), other relations not statistically significant.

Figure 2. *Profile of TNF-alpha activity in splenocyte cultures of rats with (1–3) week obstructive jaundice and in sham-operated controls.*



	1 week				2 weeks				3 weeks			
	Spontaneous		LPS induced		Spontaneous		LPS induced		Spontaneous		LPS induced	
No of rats	14	15	14	15	9	10	8	10	9	10	9	10
Mean Value +/−	3.301	820	7.959	2.391	1.019	1.360	3.695	2.886	778	585	1.853	1.532
SD	3.147	704	6.404	2.674	623	1.346	3.255	2.017	629	247	1.145	736

Significant differences: 1:5 (p=0.019), 1:9 (p=0.011), 3:7 (p<0.05), 3:11 (p=0.005), 1:2 (p=0.006), 3:4 (p=0.005), 1:3 (p=0.001), 2:4 (p=0.02), 5:7 (p=0.013), 6:8 (p=0.035), 9:11 (p=0.02), and 10:12 (p=0.001), 6:10 (near statistical difference), other relations not statistically significant.

Figure 3. *Profile of interleukin-6 in peritoneal exudate cell cultures of rats with (1–3)-week obstructive jaundice and in sham-operated controls.*

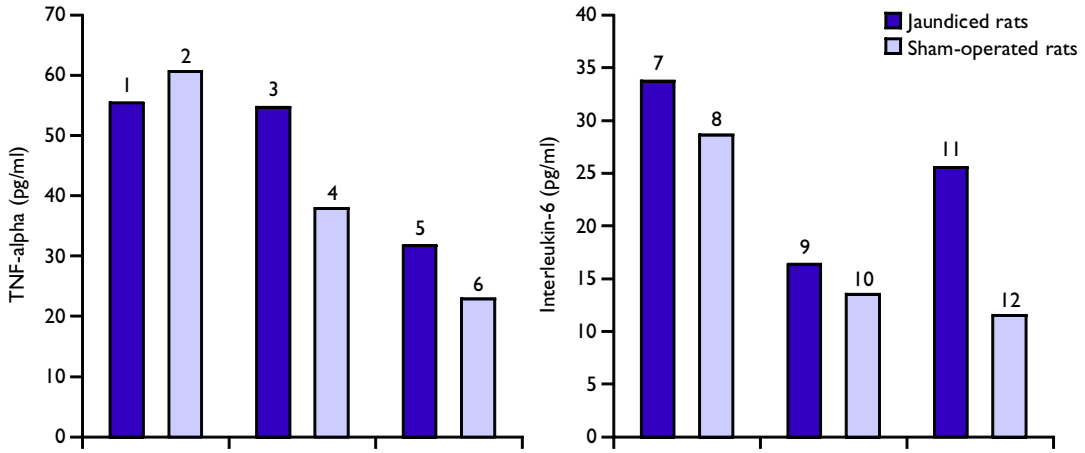
The production of IL-6 in the splenocyte cultures of both groups was similar and low at all times tested (data not shown). In addition, the differences between spontaneous and LPS-induced IL-6 production were significant. Control levels of IL-6 in naive rats were 17 and 88 pg/ml, respectively.

Levels of TNF-α and IL-6 in sera of 1–3 week OJ and sham-operated rats

Figure 4 shows that the levels of the studied cytokines in the sera differed significantly between groups. TNF-α values declined gradually from week 1 to week 3 in controls, and this decrease was statistically significant (week 1 *vs.* week 3). On the other hand, there was no change in TNF-α levels between week 1 and week 2 of OJ, and the fall in TNF-α levels at week 3 was not significant. The differences between the studied groups became even more evident in the case of IL-6. Serum IL-6 values showed also a progressive decrease in controls, while in rats with OJ there was a transient decrease in IL-6 followed by an increase, so that the final IL-6 level did not differ significantly from values at day 7. Concentrations of serum TNF-a and IL-6 in control rats were 57 and 6 pg/ml respectively.

The reticuloendothelial system dysfunction in experimental obstructive jaundice

The organ distribution of sulphur-labelled colloid is presented in Table 1. The total organ uptake of the marker was slightly lower in the liver and spleen of rats with OJ but more than twice as high in the lungs at 2 weeks, compared to controls. The specific organ uptake of colloid was lower in the liver and spleen of OJ rats but almost three times as high in the lung. The differences between total and specific organ uptake of the colloid in the spleens and livers of OJ rats was due to increased organ weight in these animals (data not shown).



	1 week	2 weeks		3 weeks		1 week	2 weeks		3 weeks		
	TNF-alpha activity of serum						Interleukin-6 activity of serum					
No of rats	15	15	16	19	8	10	15	15	16	18	8	10
Mean Value +/−	56	61	55	38	32	23	34	29	17	14	26	12
SD	21	38	40	36	12	17	32	25	7	5	8	10

Significant differences: 7:9 (p=0.03), 2:6 (p=0.009), 8:10 (p=0.01), 8:12 (p=0.01), and 11:12 (p=0.005), 1:5 and 2:4 (near statistical difference), other relations not statistically significant.

Figure 4. *Profile of TNF-alpha and interleukin-6 activities in serum of rats with (1–3)-week obstructive jaundice and in sham-operated controls.*

Table 1. *The organ distribution of colloidal sulphur labelled Technetium (TC 99 m) expressed as total organ uptake (%) and specific organ uptake (% × g^{-1})*

	Total organ uptake (%)		Specific organ uptake (% × g^{-1})	
Organ	**Sham operated rats**	**2-week obstructive jaundice**	**Sham operated rats**	**2-week obstructive jaundice**
No of rats	10	10	10	10
		(Mean value +/− standard deviation)		
Liver	83,2 +/− 1,0[1]	79,5 +/− 1,5	9,8 +/− 1,0[2]	5,9 +/− 0,3
Spleen	2,6 +/− 0,5*	2,2 +/− 0,4	4,9 +/− 0,7[3]	2,6 +/− 0,3
Lung	1,5 +/− 0,4[4]	3,8 +/− 1,2	1,3 +/− 0,3[5]	3,7 +/− 1,3
		Bilirubin level (umol/l)		
		127.4 +/− 26.4	1.08 +/− 1.3[6]	

*** – significant statistical different on level $p < 0.001$.
* – $p < 0.05$ as compared to rats with 2-week obstructive jaundice.

Discussion

In this report we have described changes in serum levels of TNF-α and IL-6 in experimental obstructive jaundice and have shown that splenocytes and PEC cultures can produce these cytokines, both spontaneously and upon induction by LPS. In addition, several parameters characterising PEC activity on day 14 of OJ were studied.

The effect of OJ was reflected by increased serum levels of IL-6 and TNF-α as compared with sham-operated control rats. Elevated serum levels of IL-6 and TNF-α were

also reported by Belemans and co-workers [18] in OJ mice. In the present study it was found that IL-6 plasma were higher in rats with OJ throughout the whole monitoring period. The differences were most evident during the early and late phase of OJ.

An analysis of TNF-α production in the population of PEC cells in both groups confirms that cells from OJ rats responded much more vigorously than control cells. Reactivity of PEC to LPS in 7–14-day OJ has been a matter of controversy. Some authors reported depressed activity of cells [3,14], but others the opposite [18,21]. Coincidentally, the increased reactivity of PEC demonstrated in our report correlated with a peak translocation of bacteria to mesenteric lymph nodes on day 7 [3]. It is, therefore, possible that the increased reactivity of PEC in OJ rats was the result of cell activation by translocating bacteria. Another characteristic feature in reactivity of PEC was the profound decrease of that function on day 14. That phenomenon was only in part due to OJ since sham-operated, control rats demonstrated a similar drop in TNF-α production. In addition, cells from both groups lost their ability to produce TNF-α on LPS induction. These effects were clearly caused by the operation itself, although the inhibition of TNF-α by PEC from OJ was more profound (taking into account 7-day values). Lastly, the regained ability of cells to respond to LPS on day 21 of OJ was somewhat unexpected. At present, explanation of that regulatory mechanism could be only speculative. Nevertheless, the inducibility of TNF-α by LPS was better in controls than OJ rats. Our data on LPS-induced TNF-α production in OJ are consistent with several studies. Puntis and Jiang [19] showed that LPS-stimulated monocytes from jaundiced patients produced significantly more TNF-α, as determined both by bioassay and radioimmunoassay. Also, in agreement with our results are data of O'Neil and colleagues [21], who demonstrated that OJ rat macrophages from macrophage-rich organs (liver, lung, spleen) produced higher amounts of TNF-α in response to LPS. The very different pattern of TNF-α production by splenocytes could be explained by different cell composition in this organ, i.e. a low macrophage content.

The differences in cell reactivity between OJ and sham-operated rats were probably best reflected by IL-6 production in PEC cultures. IL-6 production by cells from OJ rats was significantly higher than in controls and gradually decreased, whereas IL-6 production by cells from control rats was comparable on days 7 and 14. It seems, therefore, that IL-6 is a better marker of inflammation in OJ, as found in other models of inflammation [29]. Lack of a significant IL-6 response in splenocytes or of any difference between experimental groups suggests that mostly peritoneal macrophages are involved in the response to OJ-released endotoxin.

The reports on the effect of OJ on RES phagocytic activity have been conflicting, demonstrating both stimulation [31–33] and inhibition [8,34]. Later, it became clear [6] that the phagocytic activity of RES depended on the duration of OJ. We have shown that RES activity is deeply depressed on day 14 of OJ. Interestingly, this fall correlated with a profound inhibition of cytokine production by splenocytes and peritoneal cells. It seems that such a correlation does exist, since the early rise in TNF-α and IL-6 production (observed in these studies) corresponds to the rise of RES activity registered on 3–5 day of OJ [35].

The results of the study are in agreement with other reports. Pain and Drives independently reported a diminution of the clearance ability of denaturated human albumins from the serum in about 50% of OJ patients [12,35]. These observations confirmed the data of Holmberg and Tanaka, who found a decrease of liver and spleen RES activity in long-term experimental OJ and a simultaneous increase in RES activity in the lung [17,36]; Holman obtained similar results [25]. The cause of the decreased RES activity in OJ is not completely explained. It is sometimes related to the injurious effect of the bile acids or the toxic activity of conjugated bilirubin [9,17,35]. Scott-Connor suggested that the immune dysfunction of OJ may be related to systemic endotoxaemia and inappropriate elimination of enteric-derived antigens or to an increased production of cytokines [15]. The direct action of endotoxins, or their indirect effect by activation of coagulation factors, cannot be excluded [17,24,35].

In summary, we have shown for the first time that cell reactivity in OJ rats undergoes different phases; a marked depression on day 14 correlates with impaired RES activity. Significant alterations in cytokine activity throughout the monitoring time may explain discrepancies in other reports describing these parameters at different times of OJ. In addition, we showed that the operation alone may affect cell reactivity and obscure effects induced by ligation of bile ducts.

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