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ACUTE TOXICITY AND DEPRESSION OF PHAGOCYTOSIS IN VIVO BY LIPOSOMES: INFLUENCE OF LYSOPHOSPHATIDYLCHOLINE

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Summary

Small unilamellar phospholipid vesicles (liposomes), intended as drug carriers, have recently been demonstrated to reversibly depress phagocytic activity in rats when injected in a single high dose (2g of lipid per kg body weight) as revealed by the carbon clearance test. Depression of the phagocytic function was found to vary widely depending on the lipid used [M. Brandl et al., Pharm. Pharmacol. Lett.,4 (1) 1-4, 1994]. This study has now been extended in two directions: Firstly, liposomes made of the same type of lipid but different batches of raw material were compared in terms of their influence on phagocytosis as well as for their contents of impurities. The test revealed great variability of RES suppression between different batches of hydrogenated soy PC, whereas the reproducibility of the carbon clearance test was satisfactory with liposomes made of a single batch of raw material. Thin layer chromatographic analyses of the used phosphatidylcholines (PCs) and limulus tests on lipopolysaccharides revealed lysophosphatidylcholine (lysoPC) as the only impurity which showed parallels with the observed differences in phagocytosis. Secondly by "spiking" phosphatidylcholine with increasing amounts of lysoPC the latter could be proven to enhance RES depression by liposomes in a dose-dependent manner. At the same time a strong and dose-limiting increase in acute toxicity of PC vesicles was observed with increasing contents of lysoPC. However, in cholesterol-containing vesicles lysoPC-spiking did not significantly alter their behaviour, for lysoPC contents of up to 10%. Only PC/cholesterol-vesicles containing lysoPC contents as high as 15% provoked enhanced RES depression and toxicity compared to lysoPC-free vesicles. LysoPC and cholesterol in liposomes are known to play a destabilizing and stabilizing role respectively within liposomal bilayers which might influence recognition and uptake of vesicles by macrophages and thus modulation of phagocytosis.

Key Words: liposome, phagocytosis, lysophosphatidylcholine

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Main functions of the mononuclear phagocytic system are elimination of bacteria, viruses, metastases of tumours and bacterial endotoxin [1]. Phagocytes also rapidly take up colloidal and particulate drug carriers, such as liposomes [1] from the bloodstream. Accumulation of liposomes in phagocytic cells may lead to impairment or even blockade of their function. This may cause adverse effects, such as opportunistic infections, metastatic spread of tumours, or spillover of endotoxins from the gut [2, 3, 4]. Uptake of liposomes in phagocytes has long been recognized [5]. In order to achieve prolonged circulation times of liposomal drug carriers and increased levels in tissues other than liver and spleen some groups have even suggested deliberate saturation of the phagocytic activity by predosing with void vesicles or giving high liposome doses [6, 7]. Severity and duration of impairment of phagocytic activity upon administration of liposomes has not systematically been investigated. In the literature mainly data for low to moderate doses of large vesicles (MLVs or REVs) which were made of synthetic phospholipids are found [review in ref. 8]. Our interest focuses on phagocyte-suppressive effects of homogeneous, small unilamellar vesicles (SUVs) as these liposomes appear most favourable as intravenous drug carriers. In a previous study [9] these (empty) SUV preparations made of phosphatidylcholines from different sources (natural egg yolk and soy bean and their hydrogenated derivates) were found to suppress phagocytic activity significantly three hours after administration. The observed effect varied widely with the lipid(s) used. Analyses of of the used phosphatidylcholines gave hints that impurities might play a role in depression of the phagocytic activity by liposomes. In the present study therefore a twofold approach was chosen: Firstly, liposomes made of several batches of one type of phospholipid, hydrogenated soy PC, were tested for their carbon clearance depression and results were compared with purity analysis data. Secondly, one of the prominent impurities found, lysoPC, was admixed in different quantities to PC in order to find out whether these "spiked" vesicles cause enhanced RES depression.

Materials and Methods

Chemicals: Natural and hydrogenated egg and soy phosphatidylcholines (PCs) were obtained from Lipoid KG, D-Ludwigshafen and Rhone Poulenc Rorer Nattermann GmbH, D-Köln respectively. Unless indicated otherwise, lipid of a single batch was used for all consecutive experiments. LysoPCs were kindly prepared from corresponding PCs by Lipoid KG, D-Ludwigshafen; cholesterol was purchased from Croda GmbH, D-Nettetal and recrystallized from methanol before use. All other chemicals and solvents were p.a.-grade or HPLC-grade and purchased from Merck AG, D-Darmstadt. Water was freshly distilled. Analysis of impurities in PCs was carried out by quantitative thin-layer chromatography as described recently [9]. Lipopolysaccharides (LPS, endotoxins) were quantified in PCs using a modified limulus amoebocyte lysate (LAL) test. Full details of the test protocol together with results of validation will be given elsewhere [M. Brandl, manuscript in preparation]. In brief: LPS were separated from PC by ultrafiltration (Ultrasart D20, Sartorius AG, D-Göttingen) in order to avoid disturbances of the LAL test. Resuspended LPS in appropriate dilutions were incubated with LAL reagent (specific sensitivity 0.06 IU/ml, Haemachem Inc. USA-St.Louis) and analyzed for gelation after one hour at 37°C.

Preparation of small unilamellar vesicles: Small, unilamellar vesicles were prepared using a Gaulin Micron Lab 40 high-pressure homogenizer (APV Gaulin, D-Lübeck) according to the one-step method [11, 12]. In brief: dry, powdered lipids were homogenized 10 times at 70 MPa together with 0.9% NaCl solution which resulted in uniform small unilamellar vesicles. Vesicles containing cholesterol were equilibrated above 60°C after homogenisation [12]. Aseptic precautions were taken. Vesicle dispersions were filtered through 0.2 µm pore size filters (Minisart, Sartorius AG, D-Göttingen) into sterile vials and used within three days for animal tests.

Determination of carbon clearance: Depression of phagocytosis was studied in vivo using a semi-micro carbon clearance test [13]. In brief: male Wistar rats of 180-260 g body weight were heparinized and received up to 20 ml/kg of liposome dispersions containing a total of 10% (m/m) lipids or 0.9% NaCl solution (control) via slow injection into the penile vein. After 3 hours a small amount (20 mg/kg) of washed and diluted colloidal carbon (India ink, type 17, Pelikan AG, D-Hannover) was injected intravenously also into the penile vein. From blood samples taken off a canulated artery at 1-minute intervals for 12 minutes after injection carbon elimination was followed spectrophotometrically at 695 nm after hemolysis by 0.1% Na_2CO_3 solution. For evaluation of elimination constants the following equation was used (c_1, c_2 displaying plasma concentrations at time points t_1 and t_2): $k = \frac{\ln c_1 - \ln c_2}{t_2 - t_1}$

A test group consisted of 3 to 5 animals. Lower numbers of animals in some cases are due to unexepcted incidents such as sudden death. Higher numbers of animals were sometimes employed due to statistic reasons.

Results

Effect of vesicles made of different batches of hydrogenated soy PC on phagocytosis.
Vesicles made of hydrogenated soy PC had previously been found to cause most severe depression of phagocytic activity among all types of phosphatidylcholines studied [9]. In order to evaluate whether this effect is substance-specific several different batches of hydrogenated soy PC were employed for vesicle preparation and carbon clearance testing. Depression of the phagocytic activity was found to vary from batch to batch (Figure 1a). Similar tendencies were observed no matter whether vesicles made of PC only were compared or vesicles containing equimolar PC and cholesterol. The latter however always evoked less severe depression of phagocytosis. In order to study the variability of phagocytosis upon injection of similar liposomes three separate preparations of vesicles were made out of a single batch of egg PC, injected and carbon clearance determined (Fig. 1b). These tests were carried out at time points more than a year distant from each other in order to eventually detect seasonal differences.

TABLE 1
Impurities in used batches of hydrogenated soy phosphatidylcholines

type of PC	batch no.	lysoPC [%]	other phospholipids [%]	sterols [%]	"neutral lipids" arbitrary amounts	endotoxins IU/g
PPC 100H	13.03.87	1.1	n.d.	0.2	+	≤ 3.0
PPC 100H	92000300	n.d.	n.d.	n.d.	-	n.a. (≤ 12.5)[1]
PPC 100H	1623	0.5	n.d.	n.d.	-	≤ 10
PPC 100H	81000100	n.d.	n.d.	n.d.	-	≤ 4.7
S-PC-3	L11502	n.d.	n.d.	n.d.	-	≤ 4.7

Impurities in different batches of hydrogenated soy PC as revealed by quantitative thin layer chromatography and LAL test respectively. n.d. = not detectable; n.a. = not analyzed; [1] value shown in anaylsis certificate of the manufacturer. The term "neutral lipids" is used for a fraction of impurities mainly consisting of mono-, di-, and triglycerides. Exact quantities cannot be given for this fraction due to insufficient chromatographic resolution and lack of proper standards.

Seasonal differences in the results of phagocytosis were supposed by Lemperle [14], however, no systematical trials were undertaken by us in that point. The obtained results showed satisfactory consistence among the three experimental groups.

The results of quantitative thin layer chromatographic analyses of impurities in the used batches of hydrogenated soy PC along with endotoxin levels as determined by LAL tests are given in table 1. Impurities in all analyzed batches were low and well below the limits stated by the manufacturers. In hydrogenated soy PC PPC 100H (13.03.87) and PPC 100H (1623) lysoPC could be quantified (1.1 % and 0.5% respectively). In all other batches impurities were below detection limits. Endotoxin levels in all batches were low and comparable.

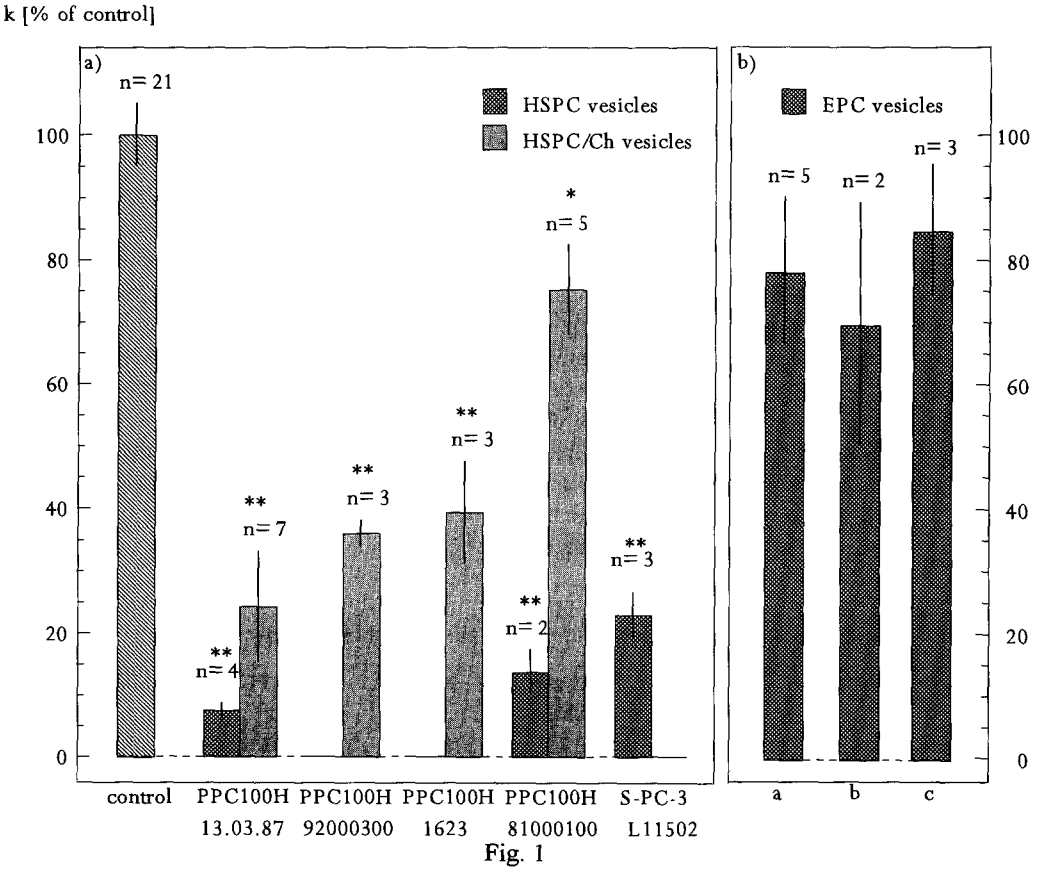

Fig. 1

Fig. 1a) Influence of vesicles made of different batches of hydrogenated soy PC on carbon clearance three hours after i.v. administration of 20 ml liposomes/kg b.w. (2000 mg of total lipid/kg b.w.) into rats. Carbon elimination constants "k" (mean ± SEM) given in % of control (saline). Results were tested for significance using Student's t-test. Significance levels: ★ $p<0.05$, ★★ $p<0.01$. HSPC = hydrogenated soy PC; EPC = egg PC; Ch = cholesterol.

Fig. 1b) Influence of three different vesicle preparations (a, b, c) made of a single batch of egg PC (EPC) on carbon clearance upon i.v. administration of 20 ml liposomes/kg b.w.. Experimental details as in Fig. 1a.

Effect of lysoPC in PC vesicles on reticuloendothelial activity.
In order to find out whether lysoPC impurities in PC vesicles contribute to the observed differences in depression of phagocytosis lysoPC was admixed in increasing quantities (% expressed as mass fraction of PC) to PC before vesicle preparation. These preparations again were used for carbon clearance studies. However, admixture of lysoPC tremendously increased acute toxicity of PC vesicles when used in a dose of 20 ml/kg i.e. 2000 mg of lipid per kg body weight. After injection of egg PC vesicles which contained 10 % of admixed lysoPC 4 out of a group of 4 rats died within half an hour. Therefore, in all subsequent tests, vesicle doses had to be reduced to 10, 5 or 2.5 ml/kg body weight corresponding to 1000, 500 and 250 mg of lipid per kg body weight respectively.

In Figure 2 depression of carbon clearance upon administration of vesicles (2.5 ml/kg) made of hydrogenated egg PC is compared which contained either 6.7 % or 10 % of admixed lysoPC.

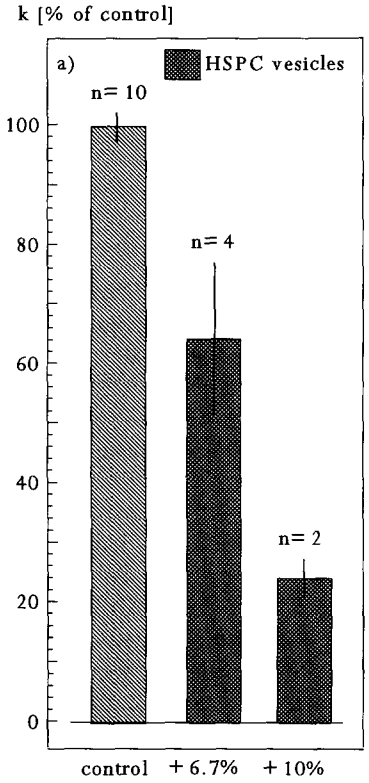

Fig. 2

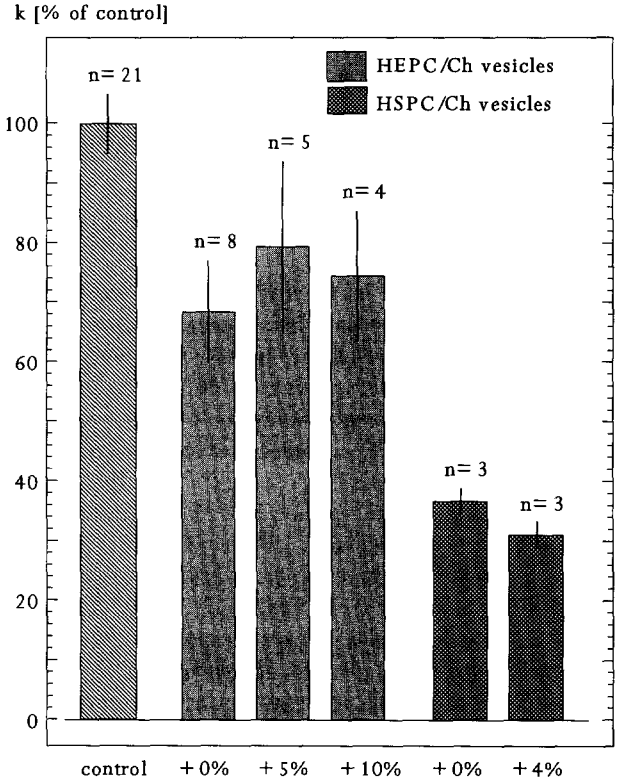

Fig. 3

Fig. 2) Influence of lysoPC "spiking" on carbon clearance depression upon i.v. administration of 2.5 ml/kg b.w. of hydrogenated egg PC vesicles (250 mg of total lipid/kg b.w.) into rats. LysoPC admixture expressed as % of amount of PC. Carbon elimination constants "k" (mean ± SEM) given in % of control (saline). HSPC = hydrogenated soy PC; HEPC = hydrogenated egg PC; Ch = cholesterol.

Fig. 3) Influence of lysoPC "spiking" in PC/cholesterol-vesicles on carbon clearance upon i.v. administration of 20 ml liposomes/kg b.w. (2000 mg of total lipid/kg b.w.) into rats. Experimental details as in Fig. 2.

The vesicles with higher lysoPC content showed enhanced depression of carbon clearance. Both preparations were more suppressive than lysoPC-free vesicles (data not shown). In Figure 3 depression of carbon clearance upon administration of vesicles (20 ml/kg) made of equimolar hydrogenated egg PC, cholesterol and 0%, 5% or 10% of lysoPC were compared to hydrogenated soy PC/cholesterol vesicles with 0% and 4% of admixed lysoPC. Results for all these cholesterol-containing vesicles were quite different from those obtained with vesicles made without cholesterol: Firstly, they could be administered in doses of 20 ml/kg without any signs of acute toxicity. Secondly, all vesicle preparations made of the same PC caused similar depression of carbon clearance regardless whether they contained lysoPC or not. However, all groups which had received vesicles made of hydrogenated soy PC showed more reduced phagocytosis compared to animals which had been given hydrogenated egg PC vesicles.

Finally admixture of very high lysoPC-contents of 15 % was tried, a value of lysoPC impurities which never had been seen when analyzing commercial batches of PC. Although 15 % lysoPC in egg PC/cholesterol vesicles was tolerated by the animals in doses of 2.5 ml, 5 ml and also 20 ml, k-values declined from 90.0 ±2.1 %, to 78.9 ±1.2 % and 12.7 ±3.9 % of control respectively (means ±SEM; n = 4 in all groups). One rat of the 20 ml group died before the end of the experiment. The result of the 20 ml group with 15% lysoPC differed significantly from the group which received 20 ml of egg PC/cholesterol vesicles without lysoPC (k = 77.1 ±7.2 %, mean ±SEM).

Discussion

The results demonstrate that activity of phagocytosis in rats was significantly reduced upon administration of small unilamellar liposomes made of hydrogenated soy phosphatidylcholine in high doses (up to 2000 mg of total lipid/kg body weight). The dose was chosen with respect to our ongoing interest in hemoglobin-containing liposomes which are intended for transfusion as blood substitutes in volumes corresponding to 10% (or even more) of the total blood volume [11, 12]. Void vesicles were chosen in order to avoid any influences of markers or drugs on phagocytic activity i.e. to study the mere liposome (lipid) effects. It has recently been shown that depression of carbon clearance upon injection of hemoglobin-containing liposomes may differ from that of void vesicles [15]. Depression of phagocytosis upon administration of liposomes has been demonstrated to vary with vesicle size, dose, dosing interval and type of phospholipids, as well as lipid autoxidation products and presence of lipopolysaccharides [16-20]. In the present study inhibition of phagocytosis was found to show a remarkable variability when hydrogenated soy PC of differnt batches was employed whereas results obtained by repeated testing of liposomes made of a single batch of egg PC were satisfactorily consistent. For vesicles which contained besides PC also equimolar amounts of cholesterol a similar pattern of decrease in phagocytosis among various batches was observed which however was less pronounced. Liposomes made of PPC 100H (13.03.87) which in a previous study [9] had been found to cause the strongest depression of carbon clearance among a variety PCs of different source and saturation degree here also exceeded depression of phagocytosis of all other tested batches of hydrogenated soy PC.

Among all impurities detected in PCs only lysoPC showed some parallels with the seen differences in carbon clearance. Therefore, lysoPC was suspected to be a RES-depressing impurity. In order to test this hypothesis, pure lysoPC was admixed to PC, and vesicles made thereof were analyzed for their effect on carbon clearance. A clear and dose-dependent depression of carbon clearance by lysoPC in vesicles could be found. However, carbon clearance depression of cholesterol-containing vesicles (made of hydrogenated egg or soy PC) was not altered significantly upon admixture of up to 10% of lysoPC. Thus, in cholesterol-containing vesicles, the

above described effect of lysoPC appeared to be widely suppressed. LysoPC as a surfactant which does not form bilayers and can cause hemolysis is expected to destabilize liposomal membranes upon incorporation [21]. As a consequence of physical destabilisation these vesicles may be phagocytized more readily. This destabilisation may, at least in certain concentrations, be compensated by cholesterol incorporation into the bilayer [17]. This matches well our observation that lysoPC did not show any effects on carbon clearance when incorporated in cholesterol-containing liposomes unless lysoPC contents exceeded 10% compared to PC. This stabilising effect of cholesterol is in good accordance with the observation that almost all cholesterol-containing vesicles caused only mild and comparable RES depression i.e. moderation of depression of phagocytosis caused by PCs.

The outstanding RES impairment evoked by hydrogenated soy PC vesicles however is unaffected by lysoPC admixture. Also the differences seen with hydrogenated soy PC vesicles made of different batches of raw material cannot fully be explained by the different contents of lysoPC. In addition lysoPC in the used batches of hydrogenated soy PC is present in much lower quantities than in previously analyzed batches of other phosphatidylcholines [9]. It therefore appears as if the outstanding severe RES depression observed with hydrogenated soy PC vesicles is not or not solely due to lysoPC impurities. Although lysoPC clearly contributes to the observed differences in depression of phagocytosis caused by different phosphatidylcholines it cannot explain all observed effects. Altogether severity of RES depression upon administration of small unilamellar vesicles depends on the lipids used. This aspect of in-vivo behaviour seems to depend on factors which also determine the physicochemical stability of phospholipid vesicles: lysoPC, a potent detergent which is known to destabilize membranes has proven to enhance RES depression. Cholsterol, which is known to condense and stabilize vesicular bilayers has been shown to reduce RES impairment. Long chain saturated PCs, which form more rigid bilayers also depress RES function to a higher extent than fluid-chain phospholipids.

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References

1. T.M. SABA, Arch. Intern. Med. 126 1031-1052 (1970).
2. M.H. LEVY and E.F. WHEELOCK, Adv. Cancer Res. 20 131-163 (1974).
3. M. GRÜN and H. LIEHR, E. Wisse and D.L. Knook (Eds.) Kupffer Cells and Other Liver Sinusoidal Cells, 437-446 (1977).
4. J. LUTZ, Int. Anaesthesiol. Clinics 23 63-93 (1985).
5. G. GREGORIADIS and E.D. NEERUNJUN, Eur. J. Biochem. 47 179-185 (1974).
6. D. LIU, A. MORI. and L. HUANG, Biochim. Biophys. Acta 1104 95-101 (1992).
7. R.L. SOUHAMI, H.M. PATEL and B.E. RYMAN, Biochim. Biophys. Acta 674 354-371. (1981).
8. T.M. ALLEN, Advanced Drug Delivery Reviews 2 55-67 (1988).
9. M. BRANDL, D. BACHMANN, A.J. AUGUSTIN, L.J.E. JÄGER and J. LUTZ, Pharm. Pharmacol. Lett. 4 1-4 (1994).
10. M. BRANDL, D. BECKER and K.H. BAUER, Drug Dev. Ind. Pharm. 15 655-669 (1989).

11. M. BRANDL, D. BACHMANN, M. DRECHSLER and K.H. BAUER, Drug Dev. Ind. Pharm. 16 2167-2191 (1990).
12. M. BRANDL, D. BACHMANN, M. DRECHSLER and K.H. BAUER, Liposome Technology 2nd Edition, Vol. 1, G. GREGORIADIS Ed., 49-66, CRC Press, Boca Raton (1993).
13. J. LUTZ and P. METZENAUER, Plügers Arch. 387 175-181 (1980).
14. G. LEMPERLE and H. HOEHLER, Therapiewoche 21 3392-3396 (1971)
15. M.C. BEACH, J. MORLEY, L. SPIRYDA and S.B. WEINSTOCK, Biomat., Art. Cells & Immob. Biotech. 20 771-776 (1992).
16. H.ELLENS, E. MAYHEW and Y.M. RUSTUM, Biochim. Biophys. Acta 714 479-485 (1982).
17. H. HOJO, Y. HOSHINO, T. KURITA and Y: HASHIMOTO, Res. Comm. Chem. Pathol. Pharmacol. 47 373-385 (1985).
18. I. FICHTNER, A. KNIEST and D: ARNDT, in vivo 6, 113-118 (1992).
19. T.M. ALLEN, L. MURRAY, S. MACKEIGAN and M. SHAH, J. Pharm. Experim. Ther. 229 267-275 (1984).
20. T.M. ALLEN, L. MURRAY, C: ALVING and J. MOE, Can. J. Physiol. Pharmacol. 65 185-190 (1987).
21. K. INOUE and T. KITAGAWA, Biochim. Biophys. Acta 363 361-372 (1974).