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Manipulation of Kupffer cells by liposome encapsulated clodronate and propamidine—synergistic and antagonistic effects of liposomal phospholipids and drugs

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Abstract

Macrophages in the liver (Kupffer cells) can be depleted by a single intravenous injection with liposome encapsulated clodronate, an anionic bisphosphonate or by liposome encapsulated propamidine, a cationic diamidine. In the present study, it was investigated whether the efficacy of the liposome mediated depletion of Kupffer cells could be enhanced by modification of the phospholipid bilayers. The efficacy of liposome mediated depletion was evaluated by comparison of the percentages of ED2-positive Kupffer cells in the rat liver, depleted by the drugs encapsulated in the liposomes at different concentrations. It is demonstrated that anionic control liposomes, containing no drug at all and composed of phosphatidylcholine (PC), phosphatidylserine (PS) and cholesterol (C) in a molar ratio of 3:3:1, reduced the percentage of ED2-positive Kupffer cells in the rat liver to about 20% of their normal numbers. At certain drug concentrations, anionic liposomes are shown to be efficacious carriers for intraphagocytic delivery of clodronate, but not for propamidine. Optimal efficacy of the latter drug was achieved by encapsulation into neutral liposomes. Omission of cholesterol or mannosylation of the phospholipid bilayers did not improve the efficacy when compared to neutral liposomes. At high concentrations of encapsulated drugs, all liposome formulations induced a nearly complete depletion of Kupffer cells ($>95\%$).

Keywords: Macrophages; Kupffer cells; Liposomes; Clodronate; Propamidine; Cell death

1. Introduction

Liposomes are efficacious vehicles for intracellular delivery of various drugs into phagocytic cells. In mammals, macrophages are multifunc-

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tional cells which are involved in the clearance of microorganisms, senescent erythrocytes, apoptotic cells and immune complexes, in the induction of acquired and natural host defense reactions and in the regulation of various functions of non-phagocytic cells (Van Rooijen et al., 1996a). In order to study the in vivo role of macrophages, the so called liposome mediated macrophage suicide approach has been developed (Van Rooijen and Sanders, 1994). It is based on liposome mediated intracellular accumulation of clodronate, an anionic bisphosphonate. Recently, it has been shown that liposome encapsulated propamidine, a cationic diamidine, can, as well, be used for depletion of liver macrophages (Kupffer cells, Van Rooijen and Sanders, 1996). Both drugs were shown to induce cell death by apoptosis when targeted into the cytoplasm of macrophages with the aid of liposomes (Van Rooijen et al., 1996b).

Liposomes used in the macrophage suicide approach are generally composed of phosphatidylcholine (PC) and cholesterol (C) in a molar ratio of 6:1, and do not exhibit an overall electrical charge for that reason (so called neutral liposomes). However, several authors have emphasized that anionic liposomes are more eagerly ingested by macrophages than their neutral or cationic counterparts (Fidler et al., 1980; Nishikawa et al., 1990). As a consequence, inclusion of PS into liposomes increases their clearance from the circulation, primarily by Kupffer cells of the liver (Liu and Liu, 1996). Although cholesterol is known to stabilize the liposomal bilayers, it has been shown to inhibit the clearance of liposomes from the circulation, presumably by inhibiting the binding of serum opsonins and consequently their phagocytosis. Actually, the highest degree of binding of blood serum proteins to liposomes and their most rapid clearance were obtained when intravenously injected liposomes did not contain any cholesterol at all (Semple et al. 1996). Incorporation of sugar ligands like mannose in the phospholipid bilayers of liposomes has been shown to accelerate their phagocytosis (Barratt et al., 1986; Garcon et al., 1988). Compared to normal liposomes containing clodronate, mannosylated clodronate liposomes caused a marked reduction of the infiltration of macrophages into the central nervous system during experimental allergic encephalomyelitis (Huitinga et al., 1990).

It was the purpose of the present study to investigate whether one of the following treatments would improve targeting of liposome encapsulated clodronate and propamidine into Kupffer cells in the rat liver. (1) Incorporation of the anionic phospholipid phosphatidylserine (PS). (2) Omission of cholesterol from liposomal bilayers. (3) Mannosylation of the liposomal bilayers. The efficacy of liposome mediated targeting was evaluated by comparison of the percentages of ED2-positive Kupffer cells, depleted by administration of the liposome encapsulated drugs (Van Rooijen and Sanders, 1996).

2. Materials and methods

2.1. Preparation and administration of liposomes

Multilamellar liposomes were prepared as described earlier (Van Rooijen and Sanders, 1994). In brief, phosphatidylcholine (Lipoid E PC, LIPOID GmbH, Ludwigshafen, Germany), phosphatidylserine (Lipoid E PS, LIPOID GmbH, Ludwigshafen, Germany) and cholesterol (Sigma, St. Louis, MO) were dissolved in chloroform in a round-bottom flask. Molar ratios of PC, PS and C were respectively, 6:0:0 (neutral liposomes without cholesterol), 6:0:1 (neutral liposomes with cholesterol), 4:2:1 (anionic liposomes with cholesterol) and 3:3:1 (strongly anionic liposomes with cholesterol). For mannosylation of liposomes, 70.9 mg PC and 10.8 mg cholesterol were dissolved in 8 ml chloroform and added to 3.6 mg *p*-aminophenyl-α-D-mannopyranoside (Sigma, St. Louis, MO) dissolved in 2 ml methanol. The molar ratio of PC:C:mannoside (7:2:1) was chosen according to Umezawa and Eto (1988) in agreement with the earlier experiments (Huitinga et al., 1990). The thin film that formed on the interior of the flask after high vacuum rotary evaporation was dispersed by gentle rotation under low vacuum for 10 min in 10 ml PBS for empty (control) PBS-liposomes or in solutions of the various drugs at different concentrations in 10 ml PBS.

After swelling, sonication and washing in PBS, the liposomes were resuspended in 4 ml PBS. Stock solutions of all drugs were made at a concentration of 0.23 M (this is the maximum concentration that can be obtained for both drugs; 0.69 M is the maximum concentration by which clodronate can be dissolved and this is the clodronate concentration that is widely used for liposome mediated macrophage depletion, Van Rooijen and Sanders, 1994). Clodronate and propamidine (isethionate) were encapsulated in liposomes in concentrations of 9, 3, 1 or 0.3 mM. Groups of four male or female Wag/rij rats (aged 8 weeks, ± 200 g) were injected with one of the three drugs in one of these concentrations. Each of the rats was intravenously injected in the tail vein with 2 ml of the liposome suspension. Clodronate was a gift of Boehringer Mannheim GmbH, Mannheim, Germany. Propamidine (isethionate) was provided by Rhone-Poulenc Rorer, Dagenham, UK.

2.2. Determination of Kupffer cells in the liver

Demonstration of macrophages in the rat liver (Kupffer cells) as well as their disappearance after administration of liposome encapsulated drugs was performed as described in earlier studies (Van Rooijen et al., 1990; Van Rooijen and Sanders, 1996). In short, rat livers were removed at 2 days after administration of liposomes, snap-frozen in liquid nitrogen and stored at −70°C. Frozen tissues were cut on a cryostat and sections of 8 μm thickness were picked up on glass slides. Sections were air-dried, fixed in pure acetone for 10 min and air-dried again. A two-step immunoperoxidase method was carried out using the monoclonal antibody ED2 for demonstration of Kupffer cells in the rat liver. ED2 recognizes resident macrophages (Dijkstra et al. 1985); in the liver, all Kupffer cells are positive for this antibody. ED2 was diluted 1:250 using PBS/BSA 0.1%. After incubation for 1.5 h at 4°C, a 1:300 dilution of rabbit anti-mouse Ig-peroxidase in PBS/BSA 0.1% was applied to the sections for 1 h at room temperature. Peroxidase was visualized with 3,3′diaminobenzidine-tetra-hydrochloride (DAB; Sigma, St. Louis, MO).

Determination of Kupffer cell numbers in the liver was performed with a normal light microscope using 200 × magnification as described earlier (Van Rooijen and Sanders, 1996). Per rat liver, numbers of Kupffer cells were determined in three different sections. Per section, Kupffer cells were counted in six areas of 0.16 mm^2 (total 0.96 mm^2). Sections in the liver and areas in the section were chosen at random. In each experiment, depletion of ED2 positive Kupffer cells was evaluated by determination of the percentage of ED2 positive cells found 2 days after administration of the liposomes. Unless stated otherwise, numbers of Kupffer cells in the untreated control group ($n = 4$) of each experiment were taken as 100%. In agreement with earlier results, about 300 ED2 positive macrophages were found per mm^2 liver tissue.

3. Results

It can be seen in Fig. 1 that neutral liposomes composed of PC and C (molar ratio 6:1) have little effect on numbers of ED2 positive Kupffer cells in the rat liver. A slight increase in cell numbers was observed. This result confirms the

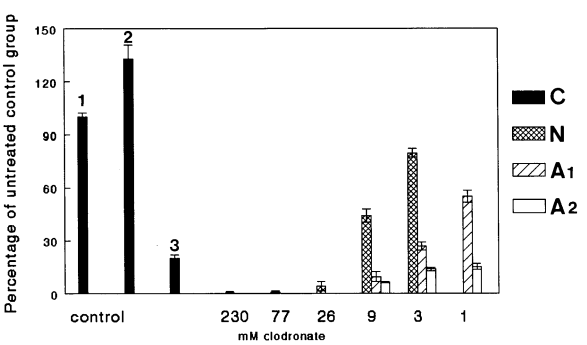

Fig. 1. Depletion of Kupffer cells in the liver by clodronate encapsulated in different concentrations in neutral liposomes or anionic liposomes containing PS. N, neutral liposomes (PC:C = 6:1); A1, anionic liposomes (PC:PS:C = 4:2:1); A2, anionic liposomes (PC:PS:C = 3:3:1). Numbers of ED2-positive Kupffer cells present 2 days after intravenous administration of liposomes were expressed as a percentage of their numbers in untreated control rats (C1 = 100%). C2, neutral liposomes (PC:C = 6:1) without clodronate; C3, anionic liposomes (PC:PS:C = 3:3:1) without clodronate.

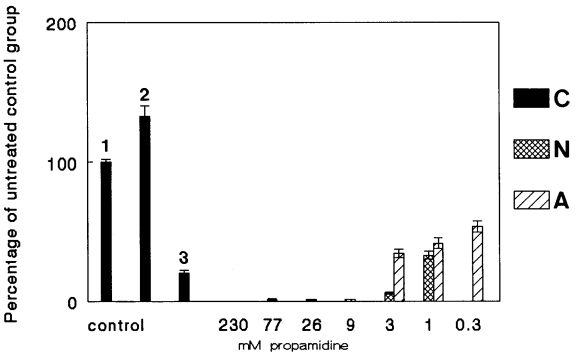

Fig. 2. Depletion of Kupffer cells in the liver by propamidine encapsulated in different concentrations in neutral liposomes or anionic liposomes containing PS. N, neutral liposomes (PC:C = 6:1); A, anionic liposomes (PC:PS:C = 3:3:1). Numbers of ED2-positive Kupffer cells present 2 days after intravenous administration of liposomes were expressed as a percentage of their numbers in untreated control rats (C1 = 100%). C2, neutral liposomes (PC:C = 6:1) without propamidine; C3, anionic liposomes (PC:PS:C = 3:3:1) without propamidine.

inert nature of such liposomes, which are widely applied in the liposome mediated macrophage suicide approach (2, 15). On the contrary, administration of liposomes in which a high extent of PS had been incorporated (PC:PS:C = 3:3:1), reduced ED2 positive Kupffer cells to about 20% of their normal numbers, confirming that incorporation of phosphatidylserine may abolish the inert character of liposomes.

Neutral liposomes, loaded with clodronate in concentrations of 26 mM or more (Fig. 1) or with propamidine in concentrations of 3 mM or more (Fig. 2), reduced the numbers of ED2 positive Kupffer cells to about 5% or less of their normal numbers. It can be seen in Fig. 1 that incorporation of PS in the phospholipid bilayers of clodronate liposomes led to a stronger reduction in the numbers of ED2 positive Kupffer cells than that achieved by neutral clodronate liposomes. This applies to both types of anionic clodronate liposomes (PC:PS:C = 4:2:1 and PC:PS:C = 3:3:1). Since anionic control liposomes (PC:PS:C = 3:3:1) in itself led to a considerable reduction in the numbers of ED2 positive Kupffer cells, the result of anionic clodronate liposomes will be caused by both the encapsulated drug and by PS incorporated in the phospholipid bilayers. In each experiment, the same amount of phospholipids was given to all animals whereas the concentrations of encapsulated clodronate were varied. As a consequence, it may be expected that at high clodronate concentrations the observed effects were mainly drug dependent, i.e. not different from the effects achieved by neutral liposomes with similar encapsulated drug concentrations. However, at low clodronate concentrations, the effects were mainly PS dependent, i.e. not different from the effects achieved by control liposomes of similar composition. It can be seen in Fig. 1 that clodronate concentrations of 9 and 3 mM encapsulated in both types of anionic liposomes result in a stronger reduction of Kupffer cell numbers than similar clodronate concentrations in neutral liposomes. On the other hand, it can be seen in Fig. 3 that these clodronate concentrations encapsulated in negative liposomes (PC:PS:C = 3:3:1) led to a stronger reduction in Kupffer cell numbers than control liposomes of similar composition. These results confirm that at these clodronate concentrations in anionic liposomes, there is a synergistic action of encapsulated clodronate and PS incorporated in the phospholipid bilayers. In the contrary, propamidine encapsulated in anionic

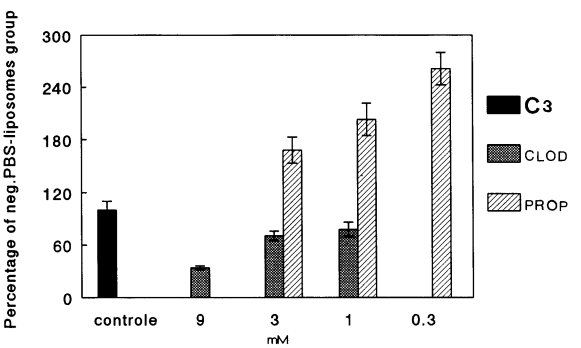

Fig. 3. Alternative reproduction of some of the results obtained by clodronate (Clod) and propamidine (Prop) encapsulated in concentrations of 9, 3, 1 and 0.3 mM in anionic liposomes containing PS (PC:PS:C = 3:3:1) and already shown in Fig. 1. This time, numbers of ED2-positive Kupffer cells present 2 days after intravenous administration of liposomes were expressed as a percentage of their numbers in control rats treated with similar liposomes without any drug encapsulated (C3 = 100%).

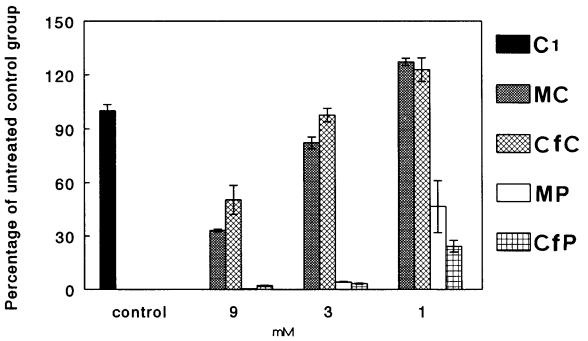

Fig. 4. Depletion of Kupffer cells in the liver by clodronate and propamidine encapsulated in different concentrations in mannosylated liposomes or cholesterol free liposomes. MC, mannosylated liposomes with clodronate; CfC, cholesterol free liposomes with clodronate; MP, mannosylated liposomes with propamidine; CfP, cholesterol free liposomes with propamidine. Numbers of ED2-positive Kupffer cells present 2 days after intravenous administration of liposomes were expressed as a percentage of their numbers in untreated control rats (C1 = 100%).

liposomes (PC:PS:C = 3:3:1) in concentrations of 3 and 1 mM had less effect on the numbers of ED2 positive Kupffer cells than similar concentrations of the drug encapsulated in neutral liposomes (Fig. 3). The latter result demonstrates that there is an antagonistic action of encapsulated propamidine and PS incorporated in the phospholipid bilayers of the liposomes.

Mannosylated liposomes and liposomes without cholesterol were about as efficacious for delivery of clodronate and propamidine into Kupffer cells as neutral liposomes (Fig. 4). Numbers of ED2 positive Kupffer cells determined after treatment of rats with neutral liposomes, mannosylated liposomes or liposomes without cholesterol were of the same order when clodronate or propamidine had been encapsulated in each of these liposomes in the same concentration. When clodronate had been encapsulated in mannosylated liposomes and liposomes without cholesterol in a concentration of 1 mM, a slight increase in the numbers of ED2 positive Kupffer cells was observed. This probably reflects the activity of the liposomal carrier itself, which, as in the case of neutral liposomes (PC:C = 6:1), induces a slight increase in numbers of ED2 positive cells. Obviously the drug had been encapsulated in a concentration too low to counteract this positive effect of the liposomes.

4. Discussion

In the present study, depletion of Kupffer cells in the rat liver by intravenously injected liposome encapsulated clodronate and propamidine was determined using a quantitative assay, based on numbers of ED2 positive Kupffer cells per surface area in liver tissues. In each experiment, the same amount of phospholipids was given to all animals whereas the concentrations of encapsulated clodronate and propamidine were varied. Compared to numbers of ED2 positive Kupffer cells in untreated animals, administration of neutral control liposomes composed of phosphatidylcholine and cholesterol (PC:C = 6:1) induced a slight increase in numbers of ED2 positive cells. A similar slight increase in numbers of ED2 positive cells was also found in rats injected with mannosylated liposomes and cholesterol free liposomes, in which clodronate had been encapsulated in a very low concentration (1 mM). This concentration appeared to be too low to affect the Kupffer cells. These three types of liposomes showed little difference in their efficacy as carriers of clodronate and propamidine into macrophages, as determined by the drug induced depletion of ED2 positive cells. Obviously, mannosylation of the liposomal bilayers, a procedure known to stimulate phagocytosis (Barratt et al., 1986; Garcon et al., 1988), as well as total omission of cholesterol, resulting in enhanced opsonization and clearance of liposomes (Semple et al. 1996), did not increase their targeting efficacy in these studies. It is concluded that there is no reason to change the phospholipid composition of the neutral liposomes (PC:C = 6:1) which are widely applied as drug carriers in the liposome mediated macrophage suicide approach (Van Rooijen and Sanders, 1994; Van Rooijen, 1995). Such liposomes are immunologically inert and their preparation is cheap and easy.

Contrary to neutral, mannosylated and cholesterol free-liposomes, anionic liposomes (PC:PS:C = 3:3:1) strongly reduced the numbers

of ED2 positive Kupffer cells, even in the absence of any encapsulated drug. This effect is probably due to the high amount of phosphatidylserine (PS) incorporated in the phospholipid bilayers of these liposomes. PS is virtually absent in the outer leaflet of cell membranes of normal cells (Op den Kamp, 1979; Williamson and Schlegel, 1994). The ratio of internal to external PS in the phospholipid bilayers of normal cells may be as high as 2000:1. This membrane phospholipid asymmetry is important in the regulation of cell functions and homeostasis. Movement of PS to the outer leaflet of the cell membrane, e.g. in aged erythrocytes, tumor cells and apoptotic cells, promotes several physiological responses eventually leading to recognition and destruction by macrophages (Bruckheimer and Schroit, 1996). PS targeted to macrophages by liposomes may affect the phospholipid asymmetry of their cell membranes during the process of endocytosis itself, e.g. if part of the liposomal PS is taken up in the outer leaflet of the cell membrane. It might also affect the macrophages after ingestion of the liposomes as a result of intracellular accumulation of liposomal PS. At the moment, the most probable explanation for the negative effect of PS inclusion in liposomes on numbers of ED2 positive Kupffer cells is a reduced expression of surface antigens on Kupffer cells, recognized by the ED2 monoclonal antibody. This in turn could be caused by a PS-induced inhibition of its production in the macrophage. Recently, it has been shown that inclusion of PS in liposomes (ratio PC:PS:C = 1:1:1) inhibits the NO production by macrophages in a dose dependent manner, whereas omission of PS (PC:C = 2:1) had no effect (Aramaki et al. 1996). Furthermore, these authors provided evidence that the inhibition was due to suppression of inducible NO synthase (iNOS) rather than by inhibition of iNOS activity itself. In other studies, it has been shown that the lipopolysaccharide induced serum levels of tumor necrosis factor (TNF) were decreased in animals treated with liposomes containing PS (Monastra and Bruni, 1994). Also in this case, the liposomal PS is thought to affect the secretion of TNF by macrophages (Brisseau et al. 1994).

Clodronate encapsulated in anionic liposomes (PC:PS:C = 3:3:1) in concentrations of 9 and 3mM led to a stronger reduction of the numbers of ED2 positive Kupffer cells than comparable concentrations of the drug in neutral liposomes (PC:C = 6:1). Obviously, at these concentrations, clodronate and anionic liposomes affect ED2 positive macrophages in a synergistic way. However, at higher concentrations of clodronate, there was no difference between the two liposome types. Enough clodronate had been encapsulated to cause a nearly complete depletion of ED2 positive cells. On the other hand, lower clodronate concentrations were not able to add to the effect already caused by liposomal PS. For the liposome mediated macrophage suicide approach, clodronate is usually encapsulated in a concentration as high as 690 mM (i.e. three times the highest concentration used in the present study, Van Rooijen and Sanders, 1994). We recommend to optimize treatment schedules for the macrophage suicide approach in each organ and animal by reducing the total dose of liposomes rather than by reducing the concentration of encapsulated drugs. So, there is no reason to replace neutral liposomes consisting of PC and C only (ratio 6:1) by anionic liposomes containing PS in their bilayers. This is more evident when propamidine is encapsulated as a macrophage depleting drug. When encapsulated in neutral liposomes (PC:C = 6:1), the Kupffer cell depleting activity of this drug exceeded that of clodronate by about a factor 10 (Van Rooijen and Sanders, 1996). However, it is shown in the present study that propamidine encapsulated in anionic liposomes (PC:PS:C = 3:3:1) and liposomal PS appeared to influence the numbers of ED2 positive Kupffer cells in an antagonistic way. It may well be that the anionic PS molecules and the cationic propamidine molecules interact in such a way that the latter are inhibited in their effect on cellular DNA.

Propamidine is a cationic antimicrobial agent belonging to the family of aromatic polyamidines (Gambari and Nastruzzi, 1994). It has been shown that polyamidines can interact with the minor groove of the DNA double helix in an integrated fashion (Edwards et al., 1992; Nunn et

al., 1993; Conte et al., 1995; Nunn and Neidle, 1995). Given the high efficacy of liposome encapsulated propamidine in the macrophage suicide approach, especially when encapsulated in simple neutral liposomes composed of PC and C only, it is anticipated that it will become an important drug in research on macrophage functions and manipulation.

Although both liposome-delivered propamidine and clodronate were shown to induce death of macrophages by apoptosis (Van Rooijen et al., 1996b), they represent quite different families of drugs. Unlike propamidine, clodronate is an anionic bisphosphonate. Its mechanism of action in the cell was speculated to depend on the intracellular depletion of crucial metal ions or ATP (Van Rooijen, 1993).

References

Aramaki, Y., Nitta, F., Matsuno, R., Morimura, Y., Tsuchiya, S., 1996. Inhibitory effects of negatively charged liposomes on nitric oxide production from macrophages stimulated by LPS. Biochem. Biophys. Res. Commun. 220, 1–6.

Barratt, G., Tenu, J.P., Yapo, A., Petit, J.F., 1986. Preparation and characterization of liposomes containing mannosylated phospholipids capable of targeting drugs to macrophages. Biochim. Biophys. Acta 862, 153–163.

Brisseau, G.F., Kresta, A., Schouten, D., Bohnen, J.M.A., Shek, P.N., Fok, E., Rotstein, O.D., 1994. Unilamellar liposomes modulate secretion of tumor necrosis factor by lipopolysaccharide-stimulated macrophages. Antimicrob. Agents Chemother. 38, 2671–2675.

Bruckheimer, E.M., Schroit, A.J., 1996. Membrane phospholipid asymmetry: host response to the externalization of phosphatidylserine. J. Leukocyte Biol. 59, 784–788.

Conte, M.R., Jenkins, T.C., Lane, A.N., 1995. Interaction of minor-groove-binding diamidine ligands with an asymmetric DNA duplex. NMR and molecular modelling studies. Eur. J. Biochem. 229, 433–444.

Dijkstra, C.D., Dopp, E.A., Joling, P., Kraal, G., 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies. Immunology 54, 589–599.

Edwards, K.J., Jenkins, T.C., Neidle, S., 1992. Crystal structure of a pentamidine-oligonucleotide complex: implications for DNA-binding properties. Biochemistry 31, 7104–7109.

Fidler, I.J., Raz, A., Fogler, W.E., Kirsh, R., Bugelsky, P., Poste, G., 1980. Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. Cancer Res. 40, 4460–4466.

Gambari, R., Nastruzzi, C., 1994. DNA-binding activity and biological effects of aromatic polyamides. Biochem. Pharmacol. 47, 599–610.

Garcon, N., Gregoriadis, G., Taylor, M., Summerfield, J., 1988. Mannose-mediated targeted immunoadjuvant action of liposomes. Immunology 64, 743–753.

Huitinga, I., Van Rooijen, N., De Groot, C.J.A., Uitdehaag, B.M.J., Dijkstra, C.D., 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J. Exp. Med. 172, 1025–1033.

Liu, F., Liu, D., 1996. Serum independent liposome uptake by mouse liver. Biochim. Biophys. Acta 1278, 5–11.

Monastra, G., Bruni, A., 1994. Decreased serum level of tumor necrosis factor in animals treated with lipopolysaccharide and liposomes containing phosphatidylserine. Lymphokine Cytokine Res. 11, 39–43.

Nishikawa, K., Arai, H., Inoue, K., 1990. Scavenger receptor mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. J. Biol. Chem. 265, 5226–5231.

Nunn, C.M., Jenkins, T.C., Neidle, S., 1993. Crystal structure of d(CGCGAATTCGCG) complexed with proparnidine, a short-chain homologue of the drug pentamidine. Biochemistry 32, 13838–13843.

Nunn, C.M., Neidle, S., 1995. Sequence-dependent drug binding to the minor groove of DNA: crystal structure of the DNA Dodecamer d(CGCAAATTTGCG)2 complexed with propamidine. J. Med. Chem. 38, 2317–2325.

Op den Kamp, J.A.F., 1979. Lipid asymmetry in membranes. Ann. Rev. Biochem. 48, 47–71.

Semple, S.C., Chonn, A., Cullis, P.R., 1996. Influence of cholesterol on the association of plasma proteins with liposomes. Biochemistry 35, 2521–2525.

Umezawa, F., Eto, Y., 1988. Liposome targeting to mouse brain: mannose as a recognition marker. Biochem. Biophys. Res. Commun. 153, 1038–1048.

Van Rooijen, N., Kors, N., Van den Ende, M., Dijkstra, C.D., 1990. Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome encapsulated dichloromethylene diphosphonate. Cell Tissue Res. 260, 215–222.

Van Rooijen, N., 1993. Extracellular and intracellular action of clodronate in osteolytic bone diseases: a hypothesis. Calcif. Tissue Intern. 52, 407–410.

Van Rooijen, N., Sanders, A., 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods 174, 83–93.

Van Rooijen, N., 1995. Functional aspects of macrophages: studies using the liposome mediated macrophage suicide approach. In: Puisieux, F., Couvreur, P., Delattre, J., Devissaguet, J.P. (Eds.), Liposomes: New Systems and New Trends in their Applications. Editions de Santé, Paris, pp. 711–734.

Van Rooijen, N., Sanders, A., 1996. Kupffer cell depletion by liposome-delivered drugs: comparative activity of intracellular clodronate, propamidine and ethylenediaminetetraacetic acid (EDTA). Hepatology 23, 1239–1243.

Van Rooijen, N., Wijburg, O.L.C., Van Den Dobbelsteen, G.P.J.M., Sanders, A., 1996a. Macrophages in host defense mechanisms. Curr. Top. Microbiol. Immunol. 210, 159–165.

Van Rooijen, N., Sanders, A., Van den Berg, T., 1996b. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. J. Immunol. Methods 193, 93–99.

Williamson, P., Schlegel, R.A., 1994. Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells (review). Mol. Membr. Biol. 11, 199–216.

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