Université de Montréal

Les liposomes fluides: fer de lance d'une antibiothérapie reciblée.

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Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiae Doctor (Ph.D)

option Microbiologie et Immumonlogie

Mars 1998

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée:

Les liposomes fluides: fer de lance d'une antibiothérapie reciblée.

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Thèse acceptée le : 21.10.1998

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RÉSUME

Au cours des deux dernières décennies, le traitement des patients atteints de fibrose kystique (FK) s'est grandement amélioré. Ces progrès font qu'aujourd'hui, cinquante pourcent de ces patients peuvent actuellement espérer atteindre l'âge de 30-35 ans, comparativement à 10-15 ans, trente ans plus tôt. Les infections respiratoires chroniques chez les patients FK constituent maintenant la cause majeure de mortalité en raison de l'incapacité des antibiotiques à éradiquer ce type d'infection causé généralement par des bactéries de la famille des *Pseudomonas*. Dans le but d'amplifier l'effet bactéricide des antibiotiques, notre laboratoire a développé durant les dernières années une formulation liposomale fluide qui a permis de combattre efficacement chez un modèle animal, les infections pulmonaires chroniques causées par *Pseudomonas aeruginosa*. Cette formulation est caractérisée par la faible température de transition de phase des phospholipides qui la composent, ce qui favorise à la fois la pénétration de l' antibiotique à travers la paroi de la bactérie et son pouvoir bactéricide. Le présent travail avait pour but: 1) d'évaluer l'efficacité bactéricide in vitro des liposomes fluides contre différentes souches bactériennes apparentées et non apparentées au *P. aeruginosa*, soit *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Escherichia coli* et *Staphylococcus aureus*; 2) de vérifier si l'utilisation directe de la forme lyophilisée des liposomes fluides conservait son activité bactéricide contre les bactéries cités précédemment; 3) d'évaluer si les liposomes lyophilisés, administrés sous forme d'aérosols conservaient leur efficacité bactéricide chez un modèle animal d'infection chronique à *P. aeruginosa*, 4) de définir les principaux paramètres touchant l'aérosolisation des liposomes fluides.

Les expériences réalisées lors de ce travail ont permis de démontrer l'efficacité bactéricide marquée des liposomes fluides sous leur forme liquide et sous leur forme lyophilisée. Il a été démontré chez les bactéries Gram négatif pré-citées que la forme liquide de l'antibiotique encapsulé avait un pouvoir bactéricide supérieur de 84 à 1 million de fois comparativement à l'antibiotique non encapsulé. Les tests effectués contre la souche gram positif montraient également une activité bactéricide fortement amplifiée comparativement à l'antibiotique non encapsulé. Les expériences réalisées directement à l'aide de la forme lyophilisée, c.à.d. sous la forme de poudre, ont montré une efficacité bactéricide atteignant 50 fois celle observée en présence de l'antibiotique libre contre les mêmes souches bactériennes utilisées précédemment et ceci malgré des concentrations d'antibiotique encapsulé inférieures à celles utilisées sous forme libre. L'administration par aérosols, sous forme de poudre, des liposomes fluides associés à la tobramycine, chez un modèle animal d'infection pulmonaire chronique, a permis de diminuer de 10^8 à $4{,}31 \times 10^5$ ufc le nombre de bactéries présentes dans les poumons des animaux ainsi traités comparativement aux animaux traités avec l'antibiotique non associé aux liposomes. Les études de stabilité, réalisées à partir de la forme réhydratée des liposomes fluides, ont montré une très bonne stabilité de l'encapsulation de l'antibiotique à 4 °C et à la température de la pièce pendant la duréée totale de l'étude, soit 28 jours; à 37°C, les liposomes ont montré une stabilité comparable à celle observée à 4 °C et à la température de la pièce sur une période de 16 hrs, soit une durée suffisante pour une activité bactéricide prolongée. Les études d'aérosolisation ont indiqué que les liposomes fluides peuvent être aérosolisés sous forme de gouttelettes ayant un

diamètre compatible avec leur déposition dans le poumon profond en raison d'un diamètre inférieur à 5 μm et que leur stabilité en terme d'encapsulation, suite à leur passage dans le nébulisateur PARI LC JET est tout-à-fait acceptable, cette dernière se situant entre 55.6 et 66.2 % dans le cas des liposomes extrudés et entre 48.5 et 68.4 % dans le cas de liposomes non extrudés. Ces travaux ouvrent la voie au développement d'une antibiothérapie plus efficace pour combattre les infections pulmonaires chroniques chez tous les patients atteints d'infections pulmonaires chroniques et plus particulièrement chez ceux atteints de fibrose kystique.

REMERCIEMENTS

A mes parents, pour leur patience et leur encouragement continuel.

Au docteur Jacqueline Lagacé, ma directrice de recherche, je tiens à exprimer toute ma gratitude pour son appui, son support exceptionnel, sa grande disponibilité, sa compréhension ainsi que pour m'avoir donné la possibilité d'approfondir mes connaissances en sciences.

Je désire témoigner ma reconnaissance au personnel du Département de microbiologie et immunologie de l'Université de Montréal qui a rendu possible la rédaction de ce mémoire, et plus spécialement au personnel associé au laboratoire du Dr Lagacé pour sa collaboration. Je désire remercier plus particulièrement M. Sébastien Sachetelli qui me fut d'un grand soutien dans la réalisation de mes travaux de recherche.

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LISTE DES ABRÉVIATIONS

ADN:	Acide désoxyribonucléique
ATP:	Adénosine triphosphate
°C:	Degré Celcius
ex:	Exemple
FK:	Fibrose kystique
LPS:	Lipopolysaccharide
pb:	Paire de bases
µg:	Microgramme
µl:	Microlitre
µmole:	Micromole
µm:	Micromètre
nm:	Nanomètre
Ml:	Millilitre
P.A.:	*Pseudomonas aeruginosa*
PBS:	Tampon phosphate buffer salin
PL:	Phospholipides
CMI:	Concentration minimale inhibitrice
C.F.T.R.:	Cystic fibrosis transmembrane regulator

INTRODUCTION

INTRODUCTION

Il y a trente ans, quand un enfant était atteint de fibrose kystique (FK), il avait à peine 50 % de chances d'atteindre l'âge de 16 ans alors qu'aujourd'hui la possibilité est de 30-35 ans. Cette augmentation de la longévité s'accompagne également d'une augmentation importante de la qualité de vie. La fibrose kystique est causée par une mutation du gène CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) qui affecte le passage des ions chlore et sodium à travers la membrane des cellules épithéliales de certains organes. Cette anomalie, a comme conséquence la déhydratation du mucus sécrété qui s'épaissit favorisant ainsi les infections pulmonaires. Ces infections pulmonaires et plus particulièrement celles causées par *Pseudomonas aeruginosa*, un pathogène opportuniste, sont responsables de plus de 95 % des décès chez les patients atteints de fibrose kystique. L'antibiothérapie traditionnelle s'avère impuissante à éradiquer les infections causées par les *Pseudomonas* en raison surtout de l'imperméabilité de la membrane externe de la bactérie aux antibiotiques et de ses sécrétions d'exopolysaccharides muqueux qui protègent la bactérie contre les défenses immunitaires de l'hôte.

Depuis plusieurs années, notre équipe tente de mieux comprendre les problèmes immunologiques des patients atteints de fibrose kystique et de trouver des solutions au problème de résistance du *Pseudomonas* aux antibiotiques. Des travaux publiés en 1994 (Omri *et al.*, 1994) ont démontré qu'il était possible d'augmenter de façon marquée le temps de persistance de la tobramycine dans l'environnement pulmonaire suite à l'administration d'antibiotique encapsulé dans des liposomes et d'en diminuer la toxicité. Ces travaux réalisés à l'aide de vésicules de type rigide qui présentaient une

température de transition de phase (T_c) élevée c'est à dire supérieure à la T° de l'organisme, n'ont pas démontré un effet bactéricide supérieur à celui observé suite à l'administration de l'antibiotique libre malgré une persistance intrapulmonaire fortement prolongée. Dans le but de tenter d'augmenter l'efficacité bactéricide des liposomes, nous avons alors développé différents types de formulations liposomales dont certaines comportaient une T_c inférieure à la température de l'organisme (Beaulac *et al.*, 1997). Une de ces formulations, composée de dipalmitoyl phosphatidylcholine (DPPC) et de dimyristoyl phosphatidylglycerol (DMPG) associée à la tobramycine, a permis de maintenir de facon satisfaisante la persistance de l'antibiotique, la diminution de sa toxicité tout en augmentant de facon marquée son efficacité bactéricide (Beaulac *et al.*, 1996). Cette formulation liposomale testée sur un modèle animal d'infection chronique à *P.aeruginosa* a entraîné l'éradication de la bactérie alors que la même dose de tobramycine sous forme libre n'a démontré aucune efficacité bactéricide (Beaulac *et al.*, 1996). Le présent travail visait à : 1) évaluer le potentiel des liposomes fluides sous forme liquide et/ou de poudre sèche contre d'autres agents bactériens apparentés et non apparentés au *Pseudomonas aeruginosa*; 2) développer un mode d'administration des liposomes par aérosols et le tester sur un modèle animal; 3) de définir les principaux paramètres touchant l'aérosolisation des liposomes fluides.

Cette thèse présentée par articles, comporte deux publications qui démontrent l'efficacité *in vitro* de la tobramycine encapsulée dans une formulation liposomale fluide contre les bactéries suivantes: *Pseudomonas aeruginosa* (PA), *Burkholderia cepacia* (BC), *Stenotrophomonas maltophilia* (SM), *Escherichia coli* (EC) et *Staphylococcus aureus* (SA). Ces études *in vitro* ont été réalisées à l'aide

d'une préparation liposomale liquide (Beaulac *et al.*, 1998) et d'une préparation liposomale sous forme de poudre sèche (Beaulac *et al.*, 1998a, soumis). Une troisième publication démontre l'efficacité bactéricide de la même formulation liposomale administrée par aérosols sous forme de poudre sèche (dry powder) sur un modèle animal d'infection chronique à *Pseudomonas aeruginosa* (souche muqueuse) (Beaulac *et al.*, 1998 b, soumis). Finalement, une étude présentée en annexe, réalisée en collaboration avec une équipe dirigée par le Dr A. Coates, spécialiste des aérosols au Sick Children Hospital de Toronto, a permis de mesurer l'influence de différents paramètres affectant l'aérosolisation des liposomes dans un but thérapeutique. Les travaux présentés dans cette thèse sont protégés par un brevet américain (# US005662929A) ainsi que par un brevet international (PCT / CA / 00713) présentement en phase pays.

REVUE DE LA LITTÉRATURE

II. REVUE BIBLIOGRAPHIQUE

2.1 La fibrose kystique

La fibrose kystique (FK), ou mucoviscidose, est la maladie héréditaire mortelle la plus répandue en Amérique du nord et en Europe. Elle est causée par un gène autosomal récessif et touche principalement la population caucasienne selon une incidence de 1\2000 à 1\4000 naissances en Europe et en Amérique du Nord (Cohen, 1986). Quatre-vingt quinze pourcent des gens atteints sont de race blanche et 54 % sont des hommes. Découverte et décrite pour la première fois en 1938 par le Dr Anderson (Anderson, 1938) pour ses manifestations intestinales, cette maladie a été caractérisée de nouveau vers 1953 par le Dr Di Sant'Agnese (Di Sant'Agnese *et al.*, 1953). Ce dernier a développé un test disgnostic basé sur l'élévation de la concentration de sodium et de chlore dans la sueur des enfants FK. Encore aujourd'hui la FK se caractérise par une sécrétion anormale des ions à travers les canaux chlores des cellules épithéliales des glandes exocrines, ainsi que par le dysfonctionnement des cellules épithéliales de l'arbre respiratoire (Jallat, 1991). Cliniquement, on observe un épaississement et une déshydratation des sécrétions exocrines qui viennent obstruer les voies respiratoires aériennes supérieures et prédisposer aux infections pulmonaires répétées. Les infections chroniques accompagnées d'inflammation persistante causent la destruction progressive du tissu pulmonaire et conduisent irréversiblement à une insuffisance respiratoire (Di Sant'Agnese, 1979; Marks, 1981; Pennington *et* al., 1979). Le pronostic vital reste étroitement lié à l'insuffisance respiratoire chronique, même si la FK est une maladie générale affectant l'ensemble des épithéliums de l'organisme, avec des

incidences pathologiques sur les fonctions pancréatiques, intestinales, hépato-biliaires et génitales. Depuis les 30 dernières années, le taux de survie des patients atteints de fibrose kystique (FK) a considérablement augmenté pour atteindre dans les années 90, un âge moyen de 32 ans au Canada et aux États Unis (Corey *et* al., 1988; Fitzsimmons, 1993; Cystic Fibrosis Fondation, 1993). Elborn et ses collaborateurs (Elborn *et* al., 1991) estiment que les enfants nés dans les années 90 auront une survie moyenne de 40 ans. Cette amélioration notable a été rendue possible grâce aux nombreuses approches thérapeutiques, qui agissent à titre préventif et qui visent à diminuer les symptômes sans toutefois permettre de traiter le défaut primaire de la maladie.

2.1.1 Le gène de la Fibrose Kystique

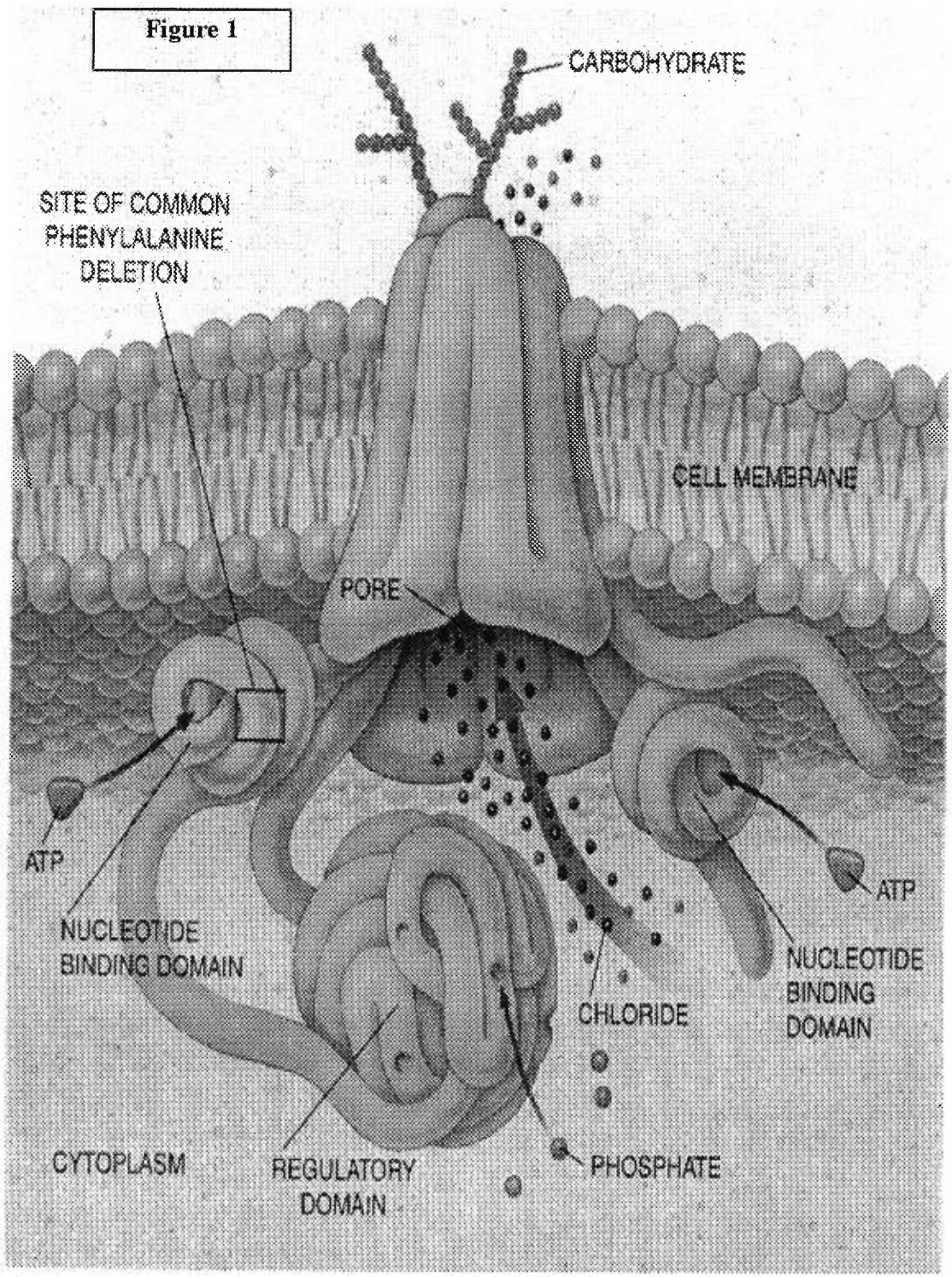
Cinquante-trois ans après la première description de la maladie, le gène "Cystic Fibrosis Transmembrane Regulator" (CFTR) responsable de ce désordre métabolique généralisé fut identifié et localisé sur le bras long du chromosomes 7 (Tsui *et al.*, 1985), puis cloné deux ans plus tard en 1989 (Tsui, 1991; Rich, 1991; Rommens *et al., 1989;* Riordan *et al.*, 1989; Kerem *et* al., 1989). Ce gène est exprimé dans plusieurs tissus affectés chez les patients atteints, comme les poumons, le pancréas, le foie, les glandes sudoripares ainsi que l'épithélium respiratoire. Le gène de la fibrose kystique code pour une protéine transmembranaire de 170 KDa sous sa forme mûre glycosilée. La protéine CFTR, qui comporte deux domaines transmembranaires à six hélices hydrophobes, est avant tout une protéine cytosolique formée de deux domaines hydrophiles NBF1 et NBF2 (*nucleotide binding fold*), sites de fixation de l'ATP et d'un domaine régulateur R, phosphorylé par des protéines kinases (Gregory *et* al.,

1990). (Fig 1). Il est important de préciser que les fonctions physiologiques de cette protéine (CFTR) sont encore incomplètement connues. Il est cependant admis que la CFTR est un canal ionique de faible conductance (7 pS), sélectif pour les anions ($Br^- > Cl^- > I^-$) et contrôlé par l'AMPc par l'intermédiaire de la phosphorylation de CFTR par une protéine kinase A au niveau du domaine R. Ce gène compte plus de 250 000 paires de bases (PB) et 24 exons. Plus de 400 mutations de ce gène ont été décrites jusqu'à aujourd'hui. La principale mutation, présente chez plus de 70 % des sujets atteints, est une mutation simple codon pour la phenylalanine en position 508. La protéine CFTR, fruit du gène du même nom, est associée directement à la régulation et au transport des ions à l'intérieur d'un canal chlore (Kartner *et al.*, 1992; Marino *et al.*, 1991; Rosenfeld *et al.*, 1992).

2.1.2 Lien entre le défaut génétique et les infections respiratoires

La plus ancienne, mais aussi la plus commune des manifestations pathologiques, est reliée à l'obstruction des bronchioles et des bronches inférieures. Le défaut primaire de cette anormalité pulmonaire est provoqué par les dysfonctions des glandes sécrétoires et subséquemment par l'inflammation qu'elles provoquent. Les glandes sous-muqueuses du tractus respiratoire constituent la principale source des sécrétions de mucus de la trachée et des bronches. La trachée d'un adulte sain contient environ une glande sécrétoire fonctionnelle par millimètre carré de surface respiratoire. Le mucus est excrété des glandes sous-muqueuses à travers de petits conduits glandulaires ciliés, qui se déversent dans des glandes tubulaires à multiples canaux, qui elles débouchent dans un acini. Ces

Figure 1


CARBOHYDRATE
SITE OF COMMON PHENYLALANINE DELETION
CELL MEMBRANE
PORE
ATP
NUCLEOTIDE BINDING DOMAIN
ATP
NUCLEOTIDE BINDING DOMAIN
CHLORIDE
CYTOPLASM
REGULATORY DOMAIN
PHOSPHATE

acini sont caractérisés par la présence de cellules séreuses. Ces cellules séreuses sécrètent un fluide qui favorise l'expulsion du mucus vers les plus grosses ramifications de l'arbre respiratoire.

En raison de l'anomalie de la protéine CFTR, le mucus des patients atteints de FK présente des concentrations en chlore de 10 % à 40 % supérieures à la normale. Cette variation en concentration ionique provoque la deshydratation ainsi que l'augmentation de la viscosité du mucus des patients FK. Ces anormalités sécrétoires réduisent la clairance mucociliaire et encouragent la colonisation bactérienne. De plus, les cellules sécrétoires distales, en plus de favoriser l'excrétion du mucus, sécrètent normalement une gamme de protéines à effet bactéricide. Ces protéines étant quasi-inexistantes chez les patients FK, favorisent par leur absence, la colonisation bactérienne (Widdicombe 1991). Les bactéries de type *Pseudomonas* sécrètent des sous-produits (exoproduits) qui stimulent les glandes sécrétoires favorisant ainsi la colonisation pulmonaire et modifiant davantage la composition du mucus sécrétoire. Ces modifications commencent par les voies respiratoires inférieures et progressent vers les voies supérieures au cours de la maladie (Khan *et al.*, 1995; Colten, 1995). La réponse inflammatoire, principalement médiée au départ par les macrophages, favorise l'afflux de neutrophiles au niveau pulmonaire par le relargage de cytokines (Khan *et al.*, 1995). L'autolyse des polymorphonuclaires provoque un relargage de différents enzymes et le maintien des réactions inflammatoires favorisant la détérioration progressive du poumon.(Stone *et* al., 1995).

2,1,3 Colonisation pulmonaire

Chez les patients FK, la colonisation bactérienne au niveau pulmonaire apparaît relativement tôt après la naissance (Myers *et al.*, 1983; Wood, 1989). La colonisation de l'arbre respiratoire est souvent la conséquence d'une série d'infections bactériennes causées par *Staphylococcus aureus*, *Haemophylus influenzae* et aboutissant à la colonisation par *Pseudomonas aeruginosa*. La très forte colonisation par *P. aeruginosa*, (plus de 10^{8} bactéries par millilitre d'expectoration chez certains patients FK) attire une très grande quantité de granulocytes dans la lumière de l'arbre bronchique (Nev, 1983; Smith *et al.*, 1988), ce qui a pour effet de créer une augmentation de la viscosité du mucus due à la présence de fortes concentrations d'ADN (Lethem *et al.*, 1990). Certaines études démontrent même l'apparition de nouveaux récepteurs chez les patients FK facilitant l'adhérence du *Pseudomonas* à l'intérieur du poumon (Prince, 1995), ainsi que la multiplication des sites d'inflammation (Orenstein *et al.*, 1993)

Depuis quelques années, chez les patients plus âgés, *Burkholderia cepacia*, une bactérie apparentée à *Pseudomonas aeruginosa*, est isolée. La colonisation par *B. cepacia* apparaît toujours suite à la colonisation par *P. aeruginosa* (Ramphal, 1991; Aronoff *et al.*, 1991). Les caractéristiques pathologiques reliées à l'infection par *B. cepacia* sont indistinctes de l'infection causée par *P. aeruginosa*. Chez quelques patients, principalement des femmes, l'infection à *B. cepacia* est caractérisée par de forts épisodes de fièvre, de la leucocytose et de fortes pneumonies qui entraînent

une détérioration rapide des fonctions respiratoires (Thomassen *et al.*, 1985) . Un infestation fulgurante par cette bactarie peut causer la mort rapide de la patiente.

D'autres micro-organismes anaérobiques et aérobiques tels des mycobactéries non tuberculisantes (Aitken, 1991), des virus, tel le virus respiratoire syncytial (Ray, 1991), des bactéries gram positives et gram négatives ont été répertoriées (Bellon *et al.*, 1991), mais ce sont les infections de type *Pseudomonas* qui sont encore aujourd'hui la majeure cause de morbidité et de mortalité chez les patients atteints de FK (Wood , 1997).

2.2 *Pseudomonas aeruginosa* et *Burkholderia cepacia*

P. aeruginosa et B. cepacia sont des bactéries opportunistes, gram négatives, mobiles grâce à une flagelle polaire, et se développant généralement dans des conditions d'aérobie stricte. Morphologiquement, elles apparaissent sous forme de bâtonnets qui mesurent 0,5 à 1 mm par 1 à 5 mm de long, et ne forment pas de spores. Ce sont des bactéries qui présentent une réaction positive au test de catalase ainsi que généralement à la cytochrome-oxydase. Sur un milieu minimal, elles présentent une morphologie plane, opaque, légèrement crémeuse et possèdent un aspect nacré à reflet métallique. La pigmentation, ainsi que l'odeur caractéristique du raisin, du taco de maïs originant de la production d'aminoacétophénone, demeurent des caractéristiques intéressantes pour le diagnostic des bactéries de type *Pseudomonas*. Grâce à leur capacité d'adaptation à différentes conditions physiques

et à leurs exigences nutritionnelles minimales, ces bactéries peuvent croître dans plusieurs environnements. *Pseudomonas* est la bactérie qui sécrète le plus grand nombre de pigments et d'exoproduits dans le milieu où elle se développe (protéases, exotoxine A, collagénases, pyoverdine, hémolysine). *P. aeruginosa*, ce germe ubiquitaire, pyocyanique se groupe en microcolonies entourées de mucus (slime) à l'intérieur du poumon. Ce mucus composé d'exopolysacharides ou alginate, multiplie par 2 ou 3 le poids de la bactérie. Il contribue ainsi à la viscosité des sécrétions bronchiques ainsi qu'à une action antiphagocytaire. Le risque d'apparition de souches mucoïdes, non typables, augmente avec l'âge du patient.

Chez les gens immunosupprimés, *P. aeruginosa*, outre des infections respiratoires, cause des infections superficielles de la peau, des ostéomyélites et des endocardites. A l'état naturel, on retrouve principalement cette bactérie dans l'eau fraîche et dans le sol. Elle est rarement responsable d'infection chez les gens en santé.

B. cepacia est principalement retrouvée au niveau du sol et comme pathogène des végétaux. Elle est associée à différentes manifestations cliniques tout comme *Pseudomonas*, mais est rarement répertoriée dans les infections nosocomiales chez les patients non FK. *B. cepacia* peut induire une détérioration respiratoire clinique aiguë alors que sa résistance aux antibiotiques est beaucoup plus grande que pour *P aeruginosa*.

2.2.1 Culture

La croissance de ces bactéries est possible à des températures variant de 5 à 42 °C, avec un optimum de croissance se situent entre 30 °C et 37 °C. Ces bactéries se cultivent facilement dans un milieu synthétique minimal simple en présence de NH4 + comme source d'azote, de glucose et d'asparagine comme source de carbone (Fick *et al.*, 1989). *P. aeruginosa* et *B. cepacia* sont des micro-organismes oxydatifs qui se développent dans des conditions d'aérobie stricte. Ils peuvent cependant croître en anaérobie en présence d'arginine, utilisant l'azote comme accepteur final d'électrons (Fick *et al.*, 1989).

2.2.2 Les pigmentations

P. aeruginosa sécrète deux types de pigments: la pyocianine et la pyoverdine. La pyocianine est un pigment bleu-vert fluorescent, dérivé de la phénazine. La production de pyocianine est largement favorisée par l'utilisation d'un milieu très bien aéré contenant peu de sels. La pyocianine est une sidérophore qui est capable de réduire le Fe + en Fe 2+ rendant ainsi le fer accessible pour la croissance de la bactérie.

P. aeurginosa est un des premiers micro-organismes dont on a démontré que les pigments présentaient un effet antibiotique (Parry *et al.*, 1976; Young *et al.*, 1984). La présence de ces pigments

peut expliquer la colonisation dominante de *P.aeruginosa* et l'inhibition des autres bactéries qui sont normalement présentes dans la flore de l'arbre respiratoire. La phénazine vient principalement inhiber la fonction ciliaire et par le fait même altérer la réponse immunitaire chez l'humain (Godfrey *et al.*, 1981; Godfrey *et al.*, 1984). La pyoverdine, pour sa part, est un pigment vert fluorescent dont la production est favorisée par le mélange de phosphate-sulfate et de glycérol.

2.2.3 Pathogénicité

La pathogénécité du *P. aeruginosa* n'est pas totalement comprise. Cependant plusieurs facteurs de virulence ont été clairement identifiés: 1) l'importance de la membrane externe de la bactérie aux points de vue de la persistance de l'infection, de l'adhésion, de la formation de microcolonies, de l'exclusion de nutriments et de sa faible perméabilité naturelle face aux agents antibactériens; la mesure directe de la perméabilité de la membrane externe indique que les taux de pénétration des antibiotiques face au *P. aeruginosa* (souche sauvage) représentent environ de 1 à 8 % de celui d'*Escherichia coli* (Angus *et al.*, 1984; Yoshimura *et al.*, 1982); 2) différents produits extra-cellulaires ou associés à la paroi de la bactérie tels la phospholipase C, l'élastase, la protéase alcaline, l'exoenzyme S, l'alginate, etc. entraînent, selon plusieurs études des effets délétères chez l'hôte (Nev, 1983; Pier, 1985). La virulence de cette bactérie est fonction de sa mucosité. Plus de 80 % des souches isolées des expectorations de patients FK sont muqueuses comparativement à moins de 3% chez des sujets non atteints (Hoiby, 1975).

2.2.4 Stratégie antibiotique

Certains antibiotiques ont une cinétique particulière: volume de distribution augmenté et clairance augmentée chez les patients atteints de FK (Omri *et al.*, 1994). Leur posologie doit donc alors être adaptée. C'est le cas de la plupart des pénicillines, céphalosporines et des aminoglycosides. Ces derniers doivent être dosés en tenant compte d'une possible oto et néphrotoxicité. Il existe donc des variations intra-individus qui obligent à des dosages très fréquents (De Groot *et al.*, 1987; Grenier *et al.*, 1987). La plupart du temps, les infections à *H. influenzae* et *S. aureus* peuvent être traités par voie orale. Les traitements anti-*P. aeruginosa* nécessitent le plus souvent la voie veineuse soit périphérique, soit par l'intermédiaire d'un cathéter central. La sévérité et la chronicité de l'infection bronchique imposent une stratégie antibiotique prenant en considération l'émergence potentielle de souches résistantes au cours des traitements successifs. Le choix des antibiotiques est également guidé par leur pénétration dans les sécrétions bronchiques.

2.3.0 Résistance aux antibiotiques

Trois mécanismes bactériens principaux viennent expliquer la résistance acquise aux antibiotiques: 1) l'inactivation ou la modification du composé par un enzyme 2) une imperméabilité accrue de la membrane externe 3) et une modification de la cible visée (Van Klingeren, 1988). Les souches de *P. aeruginosa* peuvent produire des enzymes telles la ß-lactamase (Sawai *et al.*, 1988) qui inactivent les ß-lactamines, alors que l'aminoglycoside acétyltransferase, la phosphoryltransferase et

l'adényltransferase inactivent les aminoglycosides (Van Klingeren, 1988). La résistance intrinsèque de la bactérie, qui se traduit par une faible perméabilité de la membrane externe aux différents antibiotiques joue également un rôle très important. Les lipopolysaccharides (LPS) et les OMPs "Outer Membrane Protein" pourraient être responsables de cette obstruction, que ce soit envers les ß-lactamines (Godfrey *et al.*, 1987), l'imipenem (Busher *et al.*, 1987; Stuemeister *et al.*, 1988), les quinolones (Daikos *et al.*, 1988) ou les aminoglycosides (Hancock, 1985; Norris *et al.*, 1989; Parr *et al.*, 1988). Les traitements anti-*Haemophylus* posent peu de problèmes. Une monothérapie orale de 10 à 15 jours, adaptée à l'existence d'une éventuelle β–lactamase, suffit à éradiquer ce micro-organisme. La plupart des *Staphylococcus* sont méthicilline sensibles et une monothérapie ou dans les cas les plus extrêmes, une bi-thérapie orale de 15 jours suffit en général pour diminuer voir éradiquer au moins transitoirement la bactérie. Chez certains patients atteints d'infections chroniques, il arrive que l'on doive avoir recours à l'antibiothérapie intraveineuse afin d'obtenir une amélioration clinique. Certaines études récentes favorisent un traitement préventif contre *Staphylococcus* (Weaver, 1979) tandis qu'une autre école de pensés en en désaccord démontrant que l'on favorise ainsi les phénomènes de résistance (Keflex, 1995).

A partir du moment où *P. aeruginosa* infecte le patient, le traitement antibiotique pose des problèmes particuliers. Les antibiotiques ayant quelque effet contre *P aeruginosa* n'existent que sous forme parentérale en dehors des quinolones dont l'utilisation est gênée chez l'enfant par les effets secondaires possibles sur le cartilage, donc sur la croissance de l'enfant. L'association d'antibiotiques est impérative pour diminuer le risque d'émergence de résistance. Le choix des antibiotiques est guidé par l'antibiogramme. On utilise le plus souvent une association d'un β–lactam et d'un aminoglycoside

(Foucaud *et al.*, 1990). Leurs activités sont doses dépendantes. Les aminoglycosides doivent être dosés en raison de leurs risques d'oto et de néphrotoxicité (Ristuccia *et al.*, 1992). Les aminoglycosides sont généralement administrés en une seule dose, ce qui permet l'augmentation du taux sérique sans pour autant causer trop de dommages , tandis que les β–lactams sont normalement administrés en 3 doses quotidiennes.

Il est impératif de prendre conscience de la quantité d'antibiotique qui est administrée au patient FK tout en maintenant un regard critique sur l'efficacité de cette antibiothérapie. Dans le cas d'une primo-infection à *Pseudomonas* le but est d'obtenir une éradication au moins transitoire de la bactérie (Vasquez *et al.*, 1993). Pour ce faire, on utilise une association d'antibiotiques intraveineux pendant 15 à 21 jours, suivie de nébulisation d'antibiotiques pendant 1 mois (Bellon *et al.*, 1996). Lorsque le patient passe à l'étape de l'infection chronique, c'est à dire: 1) que l'on détecte du *Pseudomonas aeruginosa* à trois reprises consécutives dans ses expectorations et 2) que l'on détecte l'existence d'au moins 2 réactions positives au anticorps anti-*Pseudomonas* dans son sérum; les cures d'antibiotiques sont alors multipliées par 2 à 4 fois ou plus annuellement. Au fur et à mesure de la progression de la maladie, l'amélioration apportée par les cures d'antibiotiques diminue et les taux de résistances augmentent. Lorsque l'infection émerge inter-cure, il n'y a pas vraiment de protocole pré-établi. Les antibiotiques en aérosols peuvent être utiles , associés ou non à une quinolone orale selon la gravité de l'infection.

2.3.1 Transplantation pulmonaire

Pour les formes très évoluées de la maladie, la transplantation pulmonaire n'est plus discutée, bien que très fortement contestée jusqu'au début des années 1990. C'est en 1984 que les premières greffes coeur-poumons ont été réalisées dans le contexte de cette maladie (Yacoub *et al.*, 1990; Cropp *et al.*, 1984). Des résultats encourageants ont très vite été enregistrés, si bien qu'aujourd'hui, la FK est la première indication pour une transplantation pulmonaire chez l'enfant. Les craintes initiales voulant que la maladie puisse se reconstituer sur les poumons greffés, c'est à dire que les problèmes sécrétoires réapparaissent dans les nouveaux poumons, se sont avérées tout-à-fait non fondées, bien que certains patients soient affligés par de nouvelles invasions bactériennes post-transplantation (Thaler *et al.*, 1997). On peut cependant considérer la transplantation pulmonaire comme une technique palliative pouvant prolonger la vie et améliorer grandement la qualité de cette dernière, mais il est important de bien garder en tête qu'il ne s'agit pas de *la solution miracle*. On peut estimer à environ 600 le nombre de patients atteints de FK ayant subi une transplantation pulmonaire à travers le monde. L'âge moyen des receveurs se situe entre 20 et 25 ans lors de l'intervention. Les taux de survie selon les différentes étude varient de façon importante en fonction du temps: de 42 à 90 % à 1 an, de 23 à 76 % à 2 ou 3 ans (Frist *et al.*, 1991; De Leval *et al.*, 1991; Madden *et* ***al.,*** 1992; Starnes *et al.*, 1992; Shennib *et al.*, 1992; Haloun *et al.*, 1996; Kotloff, 1996; Hosenpud *et al.*, 1994). Aucune différence statistiquement significative n'est retrouvée entre les résultats de la transplantation cardio-pulmonaire et ceux de la bipulmonaire. La transplantation hépatique associée ne semble pas modifier le pronostic (Couetil *et al.*, 1995). Le volume expiratoire maximum par seconde et la capacité vitale

fonctionnelle, qui se situent entre 20 et 30 % des valeurs attendues avant la greffe, atteignent 70 à 80 % au cours du deuxième semestre postopératoire. Dans les rares études effectuées, l'amélioration de la qualité de la vie a pu être mesurée chez les patients ayant passé le cap de la première année (Caine *et al.*, 1991).

2.4.0 Antibiotiques en aérosols

La possibilité d'administrer des antibiotiques directement au site de l'infection bronchique est séduisante (Sil, 1981). L'aérosolisation de l'antibiotique doit répondre à certains critères dont le diamètre des particules nébulisées qui doit se situer entre 0,5 et 5 microns, la forme de celles-ci, leur densité, leur agrégation, leur hygrométrie ainsi que leurs charges électriques. Il est aussi important de tenir compte de la configuration des voies respiratoires, une obstruction bronchique augmentant le dépôt proximal des particules. Présentement, il n'existe pas commercialement d'antibiotique sous forme d'aérosols ou sous forme de poudre à inhaler. On doit donc nébuliser des solutions injectables et s'assurer du pH et de l'osmolarité de ces dernières. Il existe trois types d'appareils permettant l'administration d'antibiotiques par voies aériennes, soit les nébulisateurs pneumatiques, les nébulisateurs ultrasoniques et les inhaleurs de particules sèches. La livraison effective au niveau pulmonaire est d'environ 10% du volume nébulisé et varie suivant le type d'appareils utilisés. Au cours d'une nébulisation, le mode ventilatoire est extrêmement important (buccale) : idéalement, on recommande l'utilisation d'un volume courant important, associé à une fréquence respiratoire lente et à des pauses inspiratoires. De nombreux antibiotiques ou molécules peuvent être utilisés:

aminoglycosides, carboxypénicillines, ceftazidinmes, colimycines. La nébulisation d'antibiotique permet de diminuer la toxicité de l'antibiotique (Schaad *et al.*, 1987; Mac Lusky *et al.*, 1989) d'où la possibilité d'augmenter la concentration de l'antibiotique dans les sécrétions bronchiques et ce, jusqu'à 10 fois la CMI de la bactérie (Ramsey *et al.*, 1993). Malgré l'intérêt suscité par l'antibiothérapie en aérosol, l'efficacité bactéricide et le contrôle infectieux demeurent des sujets qui prêtent à discussion. Selon certains auteurs, l'antibiothérapie par aérosols, comme l'antibiothérapie I.V., semblent présenter des bénéfices réels quant à l'éradication de la bactérie lors des premiers épisodes d'infections (Valerius *et al.*, 1991). Par la suite, l'antibiothérapie devient davantage un moyen de contrôle pour contrer l'apparition de manifestations trop virulentes (Hudson *et al.*, 1983). Lors d'épisodes d'exacerbation, le traitement combiné aérosols et antibiothérapie intraveineuse semble apporter un certain bénéfice en diminuant la charge bactérienne, mais le traitement n'éradique pas l'infection (Stephen *et al.*, 1983; Schaad *et al.*, 1987).

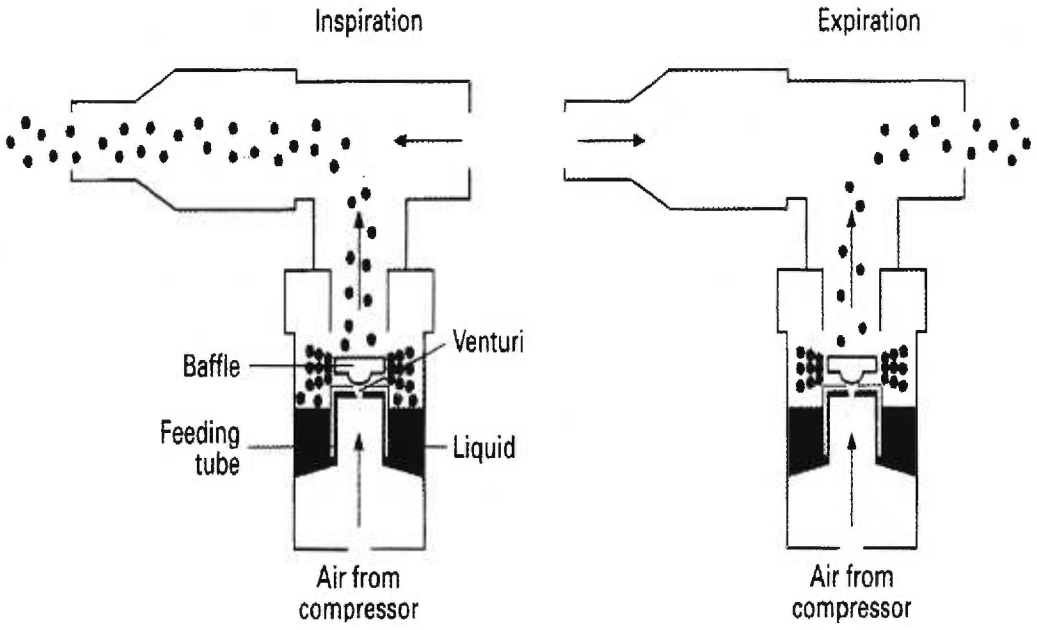
2.4.1 Nébulisateur à jet

Dans un nébulisateur à jet, le gaz (ou l'air) passe à travers une très petite ouverture qui contrôle la pression. Au niveau de l'entrée d'air, que l'on nomme Venturi, la pression diminue et la vélocité de l'air est grandement diminuée, ce qui provoque la formation d'un cône d'air. Cette diminution de vélocité et de pression est expliquée par le trajet qu'emprunte l'air avant d'entrer dans la chambre du nébulisateur. Ce cone d'air avant d'entrer se butte à une plaquette vibratoire (*baffle*) disposée vis-à-vis l'entrée de l'appareil, ce qui explique que lorsque l'air arrive avec une grande vélocité et une forte

pression, il frappe cette plaquette qui le repousse vers le bas créant ainsi une pression négative venant annuler la pression positive engendrée par l'air ou le gas fourni par le compresseur. A ce même instant, le liquide contenu dans la chambre du nébulisateur est atteint de chocs mécaniques qui sont engendrés par la vibration de la plaquette. Le liquide est alors expulsé sous forme de petits filaments suivant le principe de Bredouilla (Newman, 1989). Ces filaments se transforment en fines gouttelettes sous l'influence de la tension de surface du liquide. La première génération (atomisation) de goutelettes est de l'ordre de 15 à 500 µm de diamètre (Nerbrink *et al.*, 1994). Les gouttelettes n'atteignant pas le diamètre attendu sont retournées dans le réservoir afin d'être expulsées de nouveau du nébulisateur. La forme de la plaquette vibratoire du nébulisateur influence la forme et le diamètre des gouttelettes qu'elle génère.

Au cours des dernières années, des progrès importants ont été enregistrés en matière de moyens et de méthodes de nébulisation. Les nébulisateurs à jet de première génération ne sont pas très efficaces puisqu'ils sont à nébulisation continue et que plus de 50 % de l'aérosol est perdu durant l'expiration du patient. Des modifications ont été apportées à ce modèle pour diminuer la perte, ce qui a donné naissance aux nébulisateurs à entraînement continu de type *open vent* (exemple: Sidestream, Medic-Aid, Pagham UK). Ce type de nébulisateur produit un courant régulier et continu de goutelettes. L'ajout d'une sortie d'air sur ces modèles, favorise le maintien de la pression négative (générée par l'entrée d'air provenant du compresseur) au niveau de la plaquette vibratoire, ce qui a pour effet de standardiser davantage le diamètre des gouttelettes , de favoriser la formation de plus petites gouttelettes, et de diminuer le temps de nébulisation pour un volume donné.

Figure 2


Inspiration
Expiration
Baffle
Venturi
Feeding tube
Liquid
Air from compressor
Air from compressor

Un autre perfectionnement de la technologie a été d'ajouter une valve sur le couvercle du nébulisateur pour permettre des inspirations plus profondes, qui ne sont plus limitées au volume d'air contenu dans l'appareil ce qui permet de récupérer les gouttelettes présentes dans l'air expulsé durant l'expiration. Ce nébulisateur, le PARI LC JET, permet de générer des goutelettes uniformes de petits diamètres, de facon continue, tout en permettant d'augmenter la densité des gouttelettes par cm cube d'air inspiré.

Le nébulisateur VENTSTREAM est de même conception que le SIDESTREAM à l'exception d'une valve placée sur le coté permettant l'inspiration d'un plus grand volume d'air. Cette valve est unidirectionnelle, donc elle ne peut servir qu'au moment de l'inspiration. Ce nébulisateur est dit *open vent* assisté par la respiration. Il a pour avantage d'augmenter la densité de gouttelettes à l'inspiration, d'augmenter la volume respiratoire disponible et de permettre l'utilisation de compresseurs moins performants.

2.4.2 Nébulisateur ultrasonique

Les nébulisateurs ultrasoniques utilisent un cristal piézoélectrique à vibration rapide pour produire les gouttelettes d'antibiotique. Les vibrations transmises à la surface de la solution d'antibiotique provoquent la formation de vagues. De fines gouttelettes sont alors libérées au sommet de ces vagues et libérées sous forme de micro-gouttelettes dans l'air. Le diamètre de gouttelettes est inversement proportionnel au 2/3 du niveau de la fréquence acoustique utilisée. (Dennis *et al.*, 1992;

Newman *et al.*, 1989). Le principal avantage des nébulisateurs à ultrasons, est la rapidité avec laquelle il génère ses gouttelettes ainsi que la standardisation de celles-ci (Thomas *et al.*, 1991). Ces nébulisateurs ne seraient pas très efficaces car il semble qu'ils peuvent endommager les médicaments en raison des ultrasons émis par l'appareil ou à cause de la chaleur dégagée par ces derniers (Kholer *et al.*, 1984; Waldman *et al.*, 1987; Groth *et al.*, 1989). Ils sont cependant fortement utilisés pour les traitements utilisant les bronchodilatateurs (Thomas *et al.*, 1991) même s'ils sont plus dispendieux que les autres types d'appareils.

Figure 3

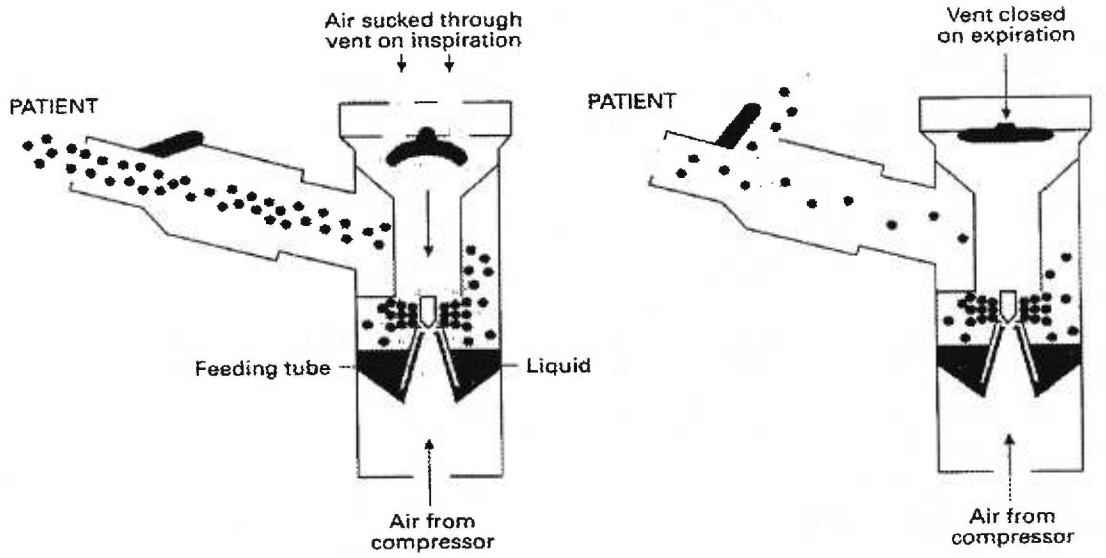

Air sucked through
vent on inspiration
PATIENT
Feeding tube
Liquid
Air from
compressor
Vent closed
on expiration
PATIENT
Air from
compressor

Figure 4

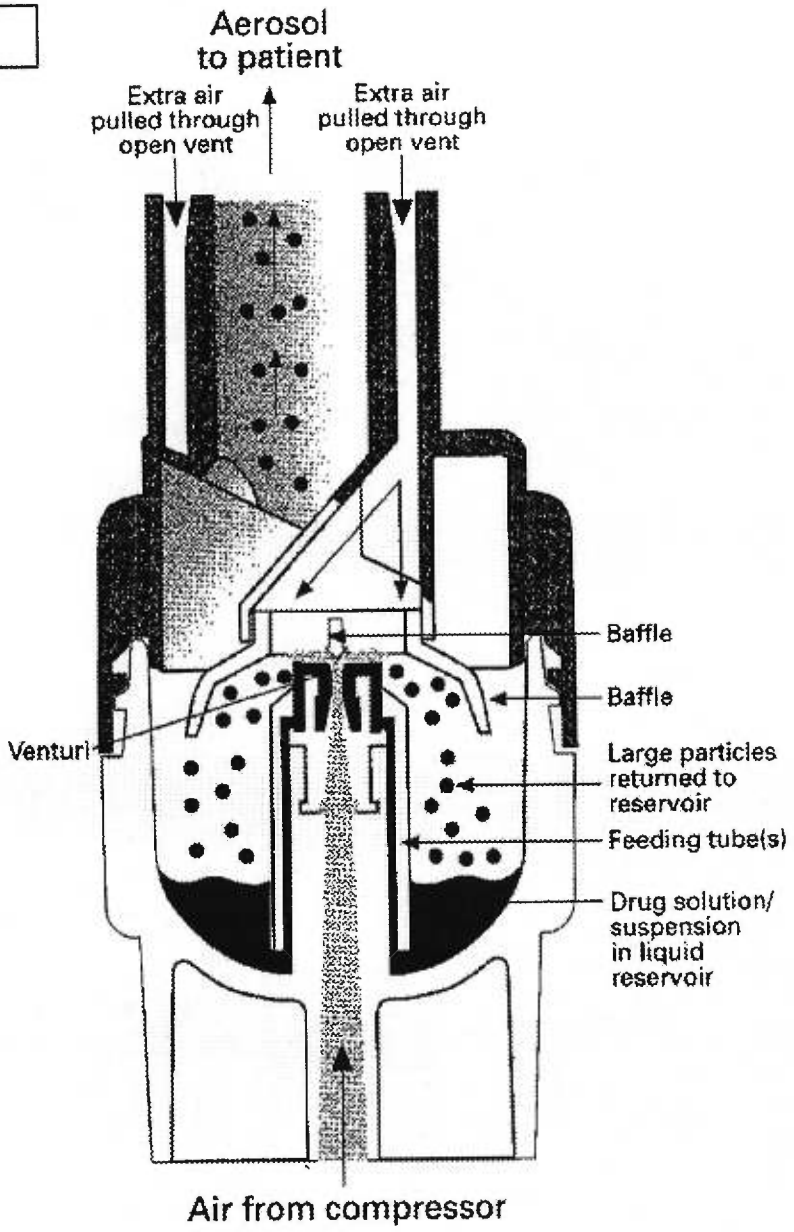
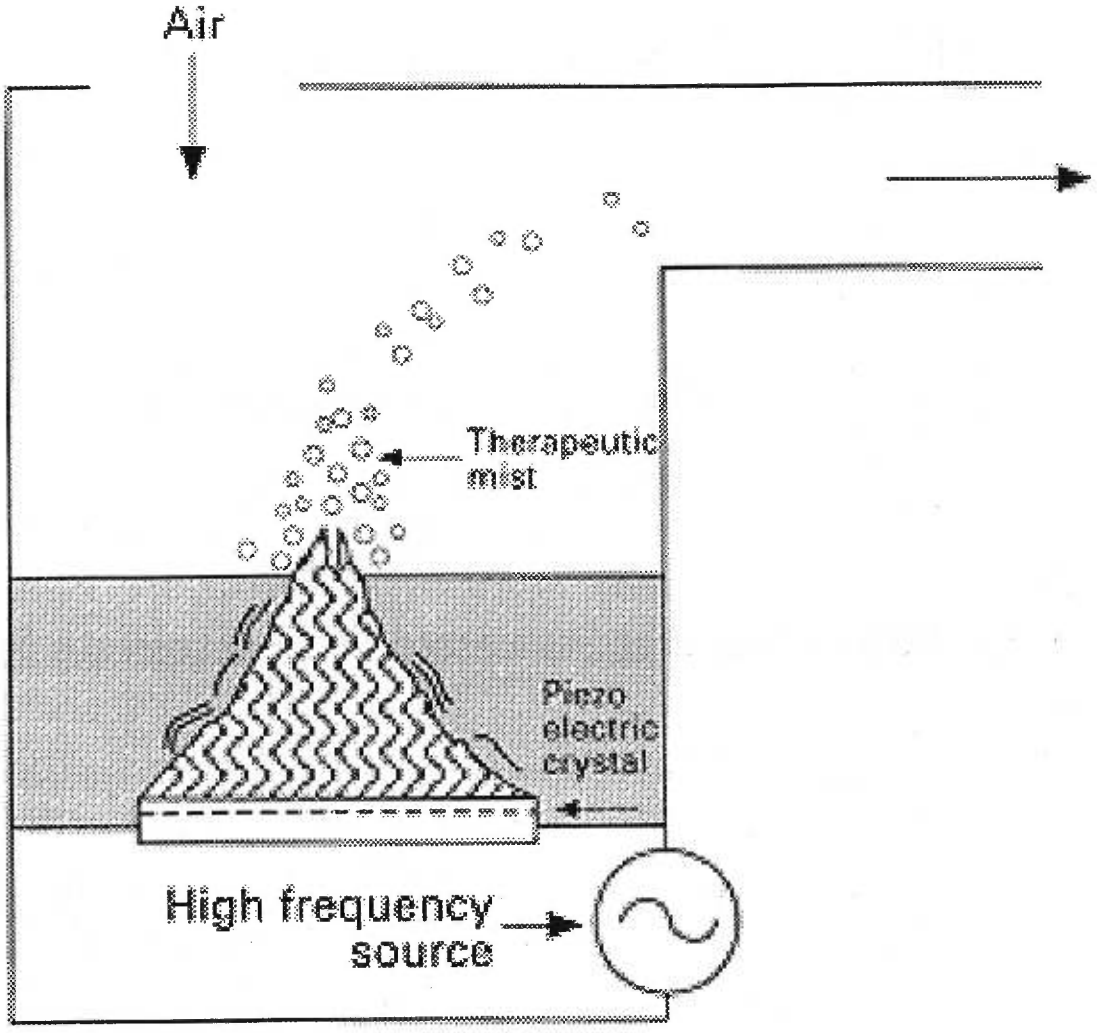

Aerosol to patient
Extra air pulled through open vent
Extra air pulled through open vent
Baffle
Baffle
Venturi
Large particles returned to reservoir
Feeding tube(s)
Drug solution/ suspension in liquid reservoir
Air from compressor

Figure 5


Air
Therapeutic mist
Piezo electric crystal
High frequency source

2.4.3 Aérosolisateur de poudre (*dry powder inhalers*)

Dans les inhalateurs de poudre, la drogue ou substance à inhaler est présentée sous une forme tout à fait non hygroscopique, souvent sous forme d'agrégats présentant un diamètre particulaire moyen d'environ 60 µm. Cette substance à inhaler est retrouvée seule ou en combinaison avec une molécule facilitant le transport ou assurant la non hygroscopicité de la drogue. Le plus grand problème des inhalateurs en poudre se situe au niveau de la dimension des particules dispersées par l'appareil, qui ont souvent un diamètre trop important pour pénétrer dans les voies respiratoires inférieures. Les molécules de transport que l'on couple à la drogue, peuvent faciliter la pénétration pulmonaire. Lors de l'inhalation, la très grande vélocité produite par l'inhalateur, permet de briser les agrégats du médicament associé aux molécules de transport. Les molécules de transport favorisent le bris des agrégats en augmentant la turbulence lors de l'inhalation, ce qui a pour conséquence de former des particules suffisamment petites pour atteindre les voies aériennes inférieures. Il est à noter que le rendement de l'inhalation des médicaments en poudre repose également sur le pouvoir inspiratoire du patient ce qui permet d'optimiser la dimension particulaire des molécules (Pederson *et al,*. 1987; Richards *et al.*, 1987) Avec un inhalateur conventionnel, environ 80 % de la dose demeure au niveau des voies respiratoires supérieures (oropharynx), 10 % est retenu à l'intérieur de l'inhalateur et environ 10 % est rendu disponible aux voies respiratoires inférieures (Pederson *et al.*, 1987; Newman *et al.*, 1981; Newman *et al.*, 1983; Borgstrom, 1993). Des inspirations très lentes, allongées et maintenues, espacées d'une pause précédant l'expiration, optimisent la technique d'inhalation et

augmentent légèrement (de 3 à 10%) le rendement de cette dernière (Pederson *et al.*, 1987; Newman *et al.*, 1983; Borgstrom *et al.*, 1993; Dolovich *et al.*, 1981; Newman *et al.*, 1981; Newman *et al.*, 1982).

Les inhalateurs les plus courants présentement sur le marché sont: Spinhaler, Rotahaler, Diskhaler et le Turbuhaler. Ces inhalateurs diffèrent en ce qui concerne leur conception et leur efficacité. Le Spinhaler est constitué d'une capsule de gélatine qui emprisonne la drogue qui est située au centre d'un rotor. Avec une technique d'inhalation optimale, 25% de la dose de départ est retenue dans la capsule et seulement de 6 à 12 % est disponible au niveau pulmonaire (Richards *et al.*, 1987; Auty *et al.*, 1987; Fuller *et al.*, 1983; Vidgren *et al.*, 1988). Le Rotahaler est aussi constitué d'une capsule de gélatine placée au centre du rotor de l'inhalateur, à la différence qu'une membrane rugueuse augmente la turbulence durant l'inhalation. La déposition intrabronchique est de 6 à 11 %, celle oropharingée est d'environ 80 % lorsque la technique d'inhalation est optimale (Vidgren *et al.*, 1988; Vidgren *et al.*, 1990; Roberts *et al.*, 1990; Zainudin *et al.*, 1990). Le Diskhaler fonctionne de manière différente. L'agent actif à délivrer est placé sous pression en présence de lactose dans une capsule d'aluminium. Lors de l'utilisation, cette dernière est brisée, expulsant la poudre contre une membrane rugueuse avec une grande vélocité. Cet inhalateur permet une déposition intrabronchiale se situent autour de 11 % (Biddiscombe *et al.*, 1991; Melchior, 1993). Le Turbuhaler, pour sa part possède un réservoir pour la poudre qui peut contenir plusieurs doses d'agent actif pur (sans additifs). Durant l'inhalation, la turbulence générée propulse l'air et le médicament en une colonne d'air sous forme de spirale contre les parois des pièces buccales rugueuses, permettant ainsi d'obtenir de plus petites particules. La déposition intrabronchiale à l'aide de cet appareil atteint les 17 à 32 %

(Borgstrom, 1993; Zainudin *et al.*, 1993; Newman *et al.*, 1989). Plusieurs autres inhalateurs sont présentement à l'étude.

2.5.0 Les Liposomes

2.5.1 Historique

En 1965, Bangman introduisait le concept de liposomes (Bangham *et al.*, 1974). Les liposomes sont de petites vésicules dont les membranes sont constituées de phospholipides (PL). Ceux-ci peuvent s'organiser de trois façons différentes dans l'eau: en micelles, en feuillets doubles ou encore en liposomes. Le type de structure formée par un PL (ou par un mélange de PL) purifié dépend de la longueur et de la saturation des chaînes acyles grasses, de la température, de la composition ionique du milieu aqueux et du mode utilisé pour disperser les PL dans la solution. Quand on agite vigoureusement dans l'eau une solution de PL, certains d'entre eux vont s'aggréger en *micelles* (structures sphériques atteignant jusqu'à 20 nm de diamètre), les chaînes grasses hydrocarbonées sont prises au piège au centre des micelles et les groupes polaires restent à la surface au contact de la phase aqueuse. Les PL peuvent aussi former spontanément des structures en feuillets symétriques. Les chaînes grasses hydrocarbonées de chaque feuillet réduisent leur contact avec l'eau en se serrant l'une contre l'autre au centre de la bicouche (Chapman, 1984). Leur compactage est stabilisé par des interactions de Vander Waals (Chapman, 1984) entre les chaînes de PL adjacents. Des liaisons

ioniques et hydrogènes stabilisent les interactions des têtes polaires avec l'eau. Une bicouche phospholipidique peut former un plan de dimension importante, allant de quelques nm à quelques mm. Les liposomes sont donc des structures bilamellaires sphériques plus grandes que des micelles et dont le centre est occupé par une phase aqueuse. Les liposomes peuvent accommoder des molécules hydrophiles dans leurs espaces aqueux et des molécules lipophiliques dans les doubles couches de lipides.

La méthode de fabrication proposée par Bangham (Bangham, 1974) produit des vésicules multilamellaires. Cette méthode consiste simplement à évaporer une solution contenant des PL dans un solvant organique afin de former un mince film dans un ballon; ce dernier est par la suite réhydraté avec une solution que l'on désire encapsuler.

Pour notre part, nous utilisons la méthode de Kirby et Gregoriadis (Kirby *et al.*, 1984) mieux connue sous le nom de méthode de déshydratation-réhydratation. Par cette méthode, à laquelle nous avons apporté des modifications, les liposomes sont lyophilisés en présence d'antibiotique ou réhydratés en présence d'un volume réduit de solution à encapsuler.

2.5.2 Caractéristiques physico-chimiques

Le choix des PL lors de la fabrication des liposomes est très important. Ces derniers influencent le taux d'encapsulation, le temps de demi-vie, le potentiel de libération et le taux de

rétention. L'utilisation de différentes combinaisons de phospholipides permet de moduler la température globale de transition de phase (Tc) des liposomes et\ou de leur fournir la charge électrique désirée par l'ajout de phospholipides chargés négativement ou positivement que l'on ajoute aux PL neutres (Beaulac *et al.*, 1997).

2.5.3 Potentiel des liposomes

Les liposomes, l'une des structure les plus étudiée dans le relargage de médicaments, peut être une alternative des plus intéressante à l'augmentation de l'efficacité des antibiotiques. L'avantage le plus connu des liposomes classiques ou rigides est qu'ils altèrent la pharmacodynamique et la biodistribution du médicament encapsulé, ce qui a pour effet d'améliorer l'efficacité du médicament et/ou d'en réduire la toxicité (Fountain *et al.*, 1985; Price *et al.*, 1992; Price *et al.*, 1994). Les liposomes contenant différents agents anti-microbiens ont démontré chez l'homme et/ou l'animal, une activité anti-infectieuse contre des protozoaires (Alvin *et al.*, 1978; Berman *et al.*, 1992), des champignons (Lopez-Berestein *et al.*, 1984; Van Etten *et al.*, 1993; Ng *et al.*, 1995) et certaines bactéries (Klemens *et al.*, 1990; Bakker *et al.*, 1995; Grayson *et al.*, 1995). Les liposomes sont administrés par voie topique (Foldvari, 1996), ophtalmique (Al-Muhammad *et al.*, 1996) ou encore par aérosols (Conley *et al.*, 1997). Les liposomes courants sont de type rigide ou classique ce qui leur confère des caractéristiques favorisant la persistance du médicament suite à leur administration et implique la nécessité de leur prise en charge par des cellules du système réticulo-endothélial (De Marie *et al.*, 1994). Ces liposomes rigides qui peuvent présenter une efficacité supérieure à l'antibiotique

libre *in vivo*, présentent généralement très peu d'efficacité *in vitro* (Bakker-Woudenberg *et al.*, 1995; Grayson *et al.*, 1995; Omri *et al.*, 1994).

L'utilisation d'une formulation liposomale de type fluide, en raison de sa température de transition de phase inférieure à la température de l'organisme, a permis de démontrer une activité bactéricide des plus efficace *in vivo* (Beaulac *et al.*, 1996) et *in vitro* (Beaulac *et al.*, 1998 a) contre des bactéries aussi variées que *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Escherichia coli* et *Staphylococcus aureus*. Ces même vésicules peuvent être administrées sous forme liquide ou encore sous forme de poudre.

2.6.0 Objectifs expérimentaux

Le choix des phospholipides qui composent les liposomes est crucial dans la détermination des propriétés physico-chimiques de ces derniers. Les liposomes fluides développés dans notre laboratoire favorisent la pénétration des antibiotiques à travers la paroi de la bactérie permettant ainsi le traitement des infections pulmonaires chroniques (Beaulac *et* al., 1996; Sachetelli *et al.*, en préparation).

Le but de ce travail était: 1) d'évaluer l'efficacité bactéricide *in* vitro des liposomes fluides contre différentes souches bactériennes apparentées ou non au *P. aeruginosa* soit *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Escherichia coli* et *Staphylococcus aureus*. 2) de vérifier si l'utilisation directe de la forme lyophilisée des liposomes fluides permettait de conserver une activité bactéricide contre les bactéries cités précédemment. 3) d'évaluer si les liposomes lyophilisés et administrés sous forme d'aérosol conservent leur efficacité bactéricide sur un modèle animal d'infection

pulmonaire chronique causé par une souche muqueuse de *P. aeruginosa*. 4) de développer une méthode pour l'administration des liposomes fluides par aérosols dans le but éventuel de traiter les patients atteints de fibrose kystique.

CHAPITRE III

JAC Manuscript No: MW 3210 REVISED

IN - VITRO BACTERICIDAL EFFICACY OF LIPOSOME-ENCAPSULATED ANTIBIOTIC IN SUB-MINIMAL INHIBITORY CONCENTRATIONS AGAINST GRAM - NEGATIVE AND GRAM - POSITIVE BACTERIA

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Running heading: Bactericidal liposome with sub - MIC antibiotic

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Recently we demonstrated that fluid rather than rigid, liposome-encapsulated tobramycin succeed in eradicating mucoid *Pseudomonas aeruginosa* in an animal model of chronic pulmonary infection (Beaulac *et al* 1996). The same liposomal formulation composed of dipalmytoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidyl-glycerol (DMPG) containing tobramycin in sub-minimal inhibitory concentrations (sub-MIC) was assayed against the following bacteria: *Pseudomonas aeruginosa, Xanthomonas (Stenotrophomonas) maltophilia, Burkholderia (Pseudomonas) cepacia, Escherichia coli* and *Staphylococcus aureus*. Exponential cultures of the above-cited clinical strains were treated with free tobramycin, liposome-encapsulated tobramycin, control liposomes without antibiotic and those combined with free tobramycin. The growth of the bacterial cells was monitored by bacterial colony counts at 0, 1, 3, 6 and 16 h after addition of antibiotic. Three hours after the addition of encapsulated antibiotic, the reduction in growth of *B. cepacia, E. coli* and *S. aureus* was of 129, 84 and 566 times respectively by comparison to cultures treated with free antibiotic. Six and 16 h after treatment, maximal reduction of growth between strains treated with liposome-encapsulated tobramycin and free tobramycin was 84, 129, 166, 10^5 and 10^4 times respectively for *P. aeruginosa,* , *B.cepacia, E. coli, X. maltophilia* and *S. aureus*. Long-term stability of liposome-associated antibiotic in the fluid liposomes was also evaluated at 4°C, at room temperature and at 37°C for a period of 28 days. Liposome-associated antibiotic was observed to be stable at 4°C and at room temperature for the whole period studied. At 37°C, an equivalent stability was observed for the first 16 h of the study. The data suggest that administration of antibiotic encapsulated in this liposomal preparation could greatly improve the management of resistant infections caused by a large range of microorganisms. Moreover, the strong bactericidal activity of the

encapsulated antibiotic at doses lower than the MIC of the strains tested cannot be explained only as a result of prolonged residence time of liposome-encapsulated tobramycin and the resulting release of entrapped antibiotic at the bacterial site. Consequently, direct interaction of chemoliposomes and bacteria, probably by a processus of fusion, must be considered to explain the marked bactericidal effect of the sub-MIC antibiotic doses used.

INTRODUCTION

The emergence of antimicrobial resistance among microorganisms is a serious medical problem attributed in large part to their misuse and overuse[1]. The development and marketing of novel antimicrobial agents have become in the last fifteen years a very expensive process[2]. This makes it imperative that new approaches to the therapy of infectious diseases be tried. Development of potentiators of known antimicrobials would be an appealing approach if it allowed increased efficacy of antibiotics and reduced doses, reducing in the same way resistance development and toxicity problems. Liposomes, the most studied colloidal drug delivery systems, could be an alternative way to improve effectiveness of antibiotics. The most well known advantages of liposomes are altered pharmacokinetics and biodistribution of the encapsulated drug molecules giving rise to improved efficacy and/or reduced toxicity of chemotherapy [3-5]. Liposome-encapsulated antimicrobial agents have been successfully used in humans and/or in animal models against protozoal[6-7], fungal[8-10] and bacterial[11-13] infections. Such experimental studies were performed with "rigid" liposomes composed primarily of natural phospholipids and cholesterol that can be polymer-coated. These liposomal formulations comprising cholesterol present necessarily a high phase transition temperature (T_C) that confers physical resistance and rigidity on the liposomes. When compared with antibiotics alone, rigid liposome-entrapped drugs may increase the therapeutic effect of the drugs in vivo while displaying generally low bactericidal activity in vitro against extracellular bacteria[12,13]. This suggests that the superior therapeutic effect of such liposome-encapsulated antibiotics results from localization and

subsequent degradation of liposomes and the resulting release of entrapped antibiotic at the infection site. Moreover, the experimental models of infections in which antimicrobial agents encapsulated in rigid liposomal formulations were successful both in vivo and in vitro studies were infections in cells of the mononuclear phagocyte system (MPS) by facultative or obligate intracellular pathogens[14,15]. In these studies, uptake of rigid liposomes by phagocytes was essential to obtain superior therapeutic effect.

Recently, the potential to achieve delivery of antibiotic to extracellular infections using rigid and fluid liposomal formulations was investigated in an experimental model of *Pseudomonas aeruginosa* chronic pulmonary infection in rats[16,17]. Eradication of the infection was observed with antibiotic encapsulated in a fluid liposomal preparation while antibiotic encapsulated in rigid liposomal preparations, free antibiotic and controls did not show any bactericidal activity[17]. The aim of the present study was to test the bactericidal efficacy of the fluid liposomal preparation: 1) against other bacteria particularly difficult to treat and related to *P. aeruginosa*, in this instance *Xanthomonas (Stenotrophomonas) maltophilia* and *Burkholderia (Pseudomonas) cepacia*; 2) against other unrelated but commun causes of infection , notably *Escherichia coli* and *Staphylococcus aureus*; 3) evaluation of long term stability of liposome-associated antibiotic at 4°C, room temperature and 37°C.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of *P. aeruginosa* (PA 429), *X. maltophilia* (C6R9) *B.* cepacia (PD-428) and *E. coli* (EC HB 101 NM 88) were isolated from the sputum of patients with cystic fibrosis (Sainte-Justine Hospital, Montréal, Québec, Canada). *S. aureus* (LSPQ 2499) a clinically derived strain was a generous gift from the Laboratoire de Santé Publique du Québec (LSPQ). Bacteria were stored at -70 °C in Brain Heart Infusion Broth (Difco Laboratories, Detroit, USA) supplemented with 20 % glycerol.

Antibiotics and susceptibility testing. Tobramycin was obtained from Eli Lilly (Toronto, Ontario, Canada). All bacterial strains were tested for antibiotic susceptibility by an agar dilution method and a broth dilution method according to the protocols of the National Commitee for Clinical Laboratory Standards[18,19]. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent (tobramycin) that inhibited the visible growth of the test bacteria. All MIC were confirmed by E-Test (AB Biodisk, Unipath, Nepean, Canada).

Liposomes. Liposome preparation consisted of dipalmytoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidyl-glycerol (DMPG) (Avanti Polar Lipids, Inc., Birmingham, USA.) in a molar ratio of 15:1 that corresponds to a global T_C of 29°C[20]. Liposomes were prepared by a dehydration-rehydration vesicle method as previously described[17]. The quantification of tobramycin in liposomal preparations was performed by high-performance liquid chromatography (HPLC) as previously described[17].

Long term release studies. The antibiotic release study over a period of 28 days was performed to measure the stability of the fluid liposomal preparation, i.e. the relative quantity of the drug found in liposomes as a function of time and temperature. DPPC/DMPG liposome-encapsulated tobramycin (888 mg) and an equivalent quantity of free tobramycin, used as control, were suspended in tubes containing 12 mL of sterile PBS and shake slowly (100 RPM/min.) at 4 °C, at room temperature and at 37 °C for 28 days. The study was done in triplicate. Samples of 500 mL were collected at 16 h, days 1, 2, 14, 21 and 28 and centrifuged at 5,000 g for 20 min (Eppendorf Centrifuge, Fisher, Montréal, Canada). After supernatant collection, the pellet was resuspended in 500 mL of PBS. Afterwards, 100 mL-samples of supernatants and resuspended pellets were submitted to HPLC analysis for tobramycin determination as previously described[17].

Experimental design. In-vitro killing curves were performed in the presence of sub-MIC concentrations of free or encapsulated tobramycin and controls made of free antibiotic combined with empty liposomes, liposomes containing PBS or PBS alone. Volumes of 250 mL of Proteose Peptone Broths no 2 (Difco Laboratories, Detroit, USA) were inoculated with a 17 h pre-culture of each bacterium to give a stock preparation containing a log-phase cell density of approximately 10^7 cfu/mL as evaluated initially by O.D. (660 nm). For more precise evaluation of the number of bacteria, culture dilutions were plated in triplicate and the number of colony forming units (cfu) was enumerated for final evaluation of the results.

All the killing tests were performed in triplicate as follows: to 29 mL of the cultures described above was added 1 mL of either free antibiotic, liposome-encapsulated tobramycin, or controls . Quantities of tobramycin contained in liposomes were first determined by HPLC analysis after

methanol extraction[17]. A second HPLC analysis was also performed after final dilutions to compare free and encapsulated antibiotic preparations. *P. aeruginosa, X. maltophilia, B. cepacia, E. coli* and *S. aureus* cultures received respectively 30.00, 1.00, 3.00, 0.75 and 1.55 mg per L of free tobramycin and 29.4, 1.00, 2.95, 0.75 and 1.57 mg per L of encapsulated tobramycin as determined by HPLC analysis. These values were lower than the respective MIC of each bacterial strain. The cultures were then incubated on a shaker at 37 °C for 16 h. Samples of 1.5 mL were collected after 0, 1, 3, 6 and 16 h of incubation. One mL was read at 660 nm and 0.5 mL was used immediately for serial dilutions in cold sterile PBS. Appropriate dilutions were plated and cultured in triplicate on Proteose Peptone no. 2 agar (Difco Laboratories). After overnight incubation at 37 °C in 5% CO_2, the number of cfu was determined.

Data analysis. All the data were analysed using the statistical program EXCEL (Microsoft Corporation), and the results were expressed as mean values +/- S.E.M.

RESULTS

Stability of antibiotic-containing liposomes. The long term release study monitored over a 28 day period at 4 °C, and at room temperature shows that in spite of their relative fluidity, the liposomes retained at these temperatures more than 66 % of the encapsulated drug until day 28 (Fig.1). About 16% of the antibiotic was found in the supernatant from the first sample collection and reached a steady state of about 21% for the whole study. The mean loss of antibiotic was approximately one third of the initial encapsulated drug which comprised the released antibiotic detected in the supernatant and a loss of about 12% due to handling. Though the results obtained at 4 °C and at room temperature were statistically comparable, larger fluctuations were observed with liposome encapsulated tobramycin maintained at room temperature (Fig.1). Analysis of the samples collected at 16 hrs from liposome preparations maintained at 37 °C, showed that the release of antibiotic was comparable for this time to the release observed at 4 °C and at room temperature. Later on, liposomes maintained at 37 °C showed sustained significant release of tobramycin during the remainder of the study (Fig. 1). At the end of the study, only 8% of the drug was still encapsulated in the liposomes incubated at 37 °C (Fig.1). Free tobramycin used as a reference showed that the measure of tobramycin by HPLC was not significantly influenced by temperature variations during the whole test period (data not shown).

In-vitro bactericidal efficacy of liposome-encapsulated versus free tobramycin. The MICs of tobramycin for *P. aeruginosa, X. maltophilia, B. cepacia, E. coli* and *S. aureus* were found to be respectively 60, 2, 5, 1.5 and 2 mg/L. As indicated in materials and methods, the doses of

encapsulated-tobramycin were adjusted to be lower than or equal to free tobramycin and lower than the MIC of the 5 bacterial strains tested. Killing curves of bacteria were evaluated by enumeration of cfu made on agar plates (Fig.2a-e). Controls made of liposomes without antibiotic and liposomes combined with free antibiotic were not included in the figures because of the superimposition of these results with PBS controls and free tobramycin.

In cultures of *B. cepacia, E. coli* and *S. aureus,* 3 h post-treatment with liposome-encapsulated antibiotic, the reduction in growth was respectively of 129, 84 and 566 times greater than in cultures treated with free antibiotic (Fig.2c, d,e). At 6 h and/or 16 h after treatment, a decrease of cfu/mL was observed for all the strains treated with liposome-encapsulated tobramycin (Fig. 2a-e) while bacterial growth reached approximately 10^8 - 10^9 cfu/mL in PBS-control cultures. Following the addition of free antibiotic, the culture growth of the *Pseudomonas* group was minimally affected whereas a considerable growth decrease was observed between 1 and 6 h for *E. coli* and *S. aureus* (Fig. 2d,e). However, the same quantity of encapsulated antibiotic showed cfu decreases of *S. aureus* and *E. coli* of more than 10^4 times and 166 times respectively comparative to free antibiotic (Fig.2e).

At 16 h post-treatment, significant increase in growth of *B. cepacia*, *E. coli* and *S. aureus* cultures treated with encapsulated antibiotic were noted; however, the cfu counts were still respectively of 35, 166 and 163 times lower than the cfu counts in cultures treated with free antibiotic (Fig.2c,d,e).

DISCUSSION

In previous studies, in vitro drug release kinetics and pulmonary retention of encapsulated antibiotic in liposomes and function of their lipid composition in terms of bilayer fluidity and charge were investigated[20]. Fluid liposomes composed of DPPC and DMPG showed modulated release of antibiotic in vitro while maintaining good pulmonary persistence of the drug. Afterwards, liposome-encapsulated tobramycin with T_C of 29 °C and 32 °C, contrarily to more rigid liposomes, was shown to eradicate mucoid *P. aeruginosa* in an animal model of chronic pulmonary infection[17]. The present in vitro study using this liposomal formulation (T_C of 29°C) and comprising tobramycin in sub-MIC concentration, showed a marked bactericidal activity not only against *P. aeruginosa* but also against all other strains tested. Broad antibacterial activity of liposomes was demonstrated by the efficacy of the encapsulated antibiotic against *E. coli* and *S. aureus* whose cell-walls are structurally very different from *Pseudomonas*.

To assure accuracy and reliability of the doses of tobramycin administered in this study, strict controls were effected by doing two independent HPLC analysis on free and encapsulated tobramycin before and after final dilutions. The results obtained following the use of sub-MIC quantities of encapsulated tobramycin against 3 related strains of the *Pseudomonas* group are striking since this group is recognized for its very low nonspecific permeability and/or the presence of an efflux pump in its external membrane[21-23]. The maximal bactericidal activity of the encapsulated antibiotic against *P.*

aeruginosa and *X. maltophilia* was observed at 6 to 16 h (Fig 2a and b). The bactericidal effect took less time to be established (between 1 and 3 h) in *B. cepacia*, *E. coli* and *S. aureus* (Fig.2c,d,e). This may be explained at least in part by the remarkable impermeability of the *Pseudomonas* outer membrane which is a major obstacle to antibiotic penetration[23]. The lipopolysaccharide side chains of *Enterobacteriacae* such as *E. coli* are crosslinked which confers an intermediate impermeability to their outer membrane compared to that of *P. aeruginosa*[24] while the peptidoglycan layer of Gram-positive bacteria generally does not pose a significant barrier to antibiotic entry[23]. This and shorter generation times[25] can also explain why these differences are seen.

The fact that the use of sub-MIC of encapsulated antibiotic succeeded in reducing the growth of bacteria by 35 to 10^5 times in comparison with cultures treated with the same quantity of free antibiotic suggests a direct interaction of liposome with bacteria. It is possible that due to the fluidity or plasticity of the DPPC-DMPG liposomal formulation, a fusion mechanism could be at work. Such fusion would result in improved passage of liposome-encapsulated antibiotic through the cell walls of Gram-negative and Gram-positive bacteria. Preliminary works that are in progress in our laboratory seem to support this hypothesis (data not shown).

The data obtained from the long term release study indicate that antibiotic-containing liposomes are relatively stable at 4°C and room temperature. Indeed, more than 66% of the antibiotic is recovered from the liposomes at the end of the 28 day period (Fig. 1). However at room temperature, some instability between encapsulation and disencapsulation seems to occur (Fig. 1). This may be explained by the fact that the T_C of the liposomes is of 29°C, which is very near the working temperature. Thus at this temperature, the transition between solid and liquid-crystalline phase could

not be very stable if we consider possible variations of room temperature. Consequently, the phospholipids seem continuously to be shifting between the two states (Fig.1). The study performed at 37°C, 8 degrees above the T_C of the liposomes, showed that until 16 h , liposomes still retained the same quantity of the drug as noted for liposomes maintained at 4°C and room temperature (Fig 1). This is very important and means that in spite of a high level of lability, liposomes can retain the drug for a sufficient period of time to allow effective interactions. This liposomal plasticity could favour integration to bacterial cell walls. The plausibility of such integration is in accordance with the fact that the liposomes, even if they contained sub-MIC tobramycin, showed at 37°C important bactericidal activity against a broad range of bacteria.

Bacteria exert antibiotic resistance mainly by preventing entry of the drug, by rapidly extruding the drug or by enzymatically inactivating the drug or altering its molecular target[23]. Recent studies suggest that mechanisms of resistance work in a synergistic way, decreasing permeability and/or the presence of membrane-associated energy driven efflux systems playing an important role in the phenomenon[23,26,27]. It is well known that in Gram-negative bacteria, enzymes exert their modification of the drug together with the barrier permeability of the outer membrane[24,28]. Low affinity to the molecular target of the drug or accelerated penetration of the drug reduce the capacity of enzymes to protect the bacteria[24]. If we consider the importance of the penetration barrier in antibiotic resistance in bacteria, the data obtained with the DPPC/DMPG liposomal formulation suggest that the liposome-encapsulated antibiotic described could not only increase the efficacy of antibiotic treatments with non-resistant strains but could also contribute to overcome bacterial resistance. Work is in progress to measure the efficacy of this liposomal formulation against multidrug resistant mutants.

ACKNOWLEDGMENT

This study was supported by a grant from the Canadian Cystic Fibrosis Foundation.

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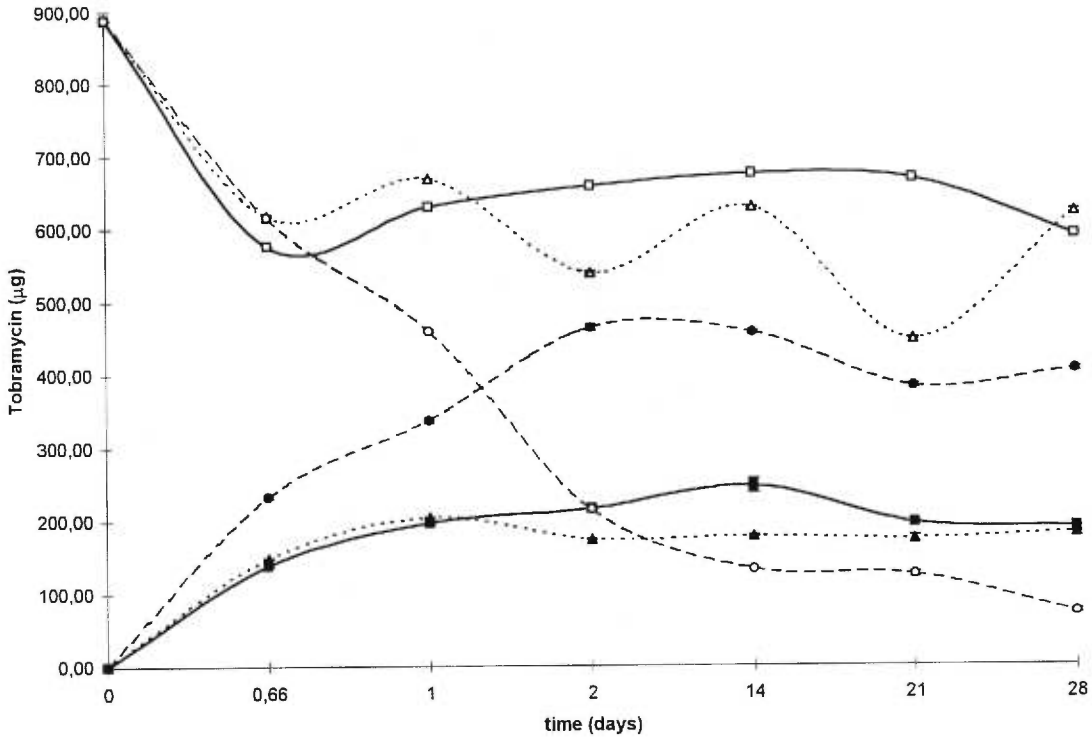
Figure 1. Stability study of fluid liposomes containing antibiotic (888 mg) still encapsulated after incubation at: 4°C (—□—) , at room temperature (··▲··), and at 37°C (–○–). Antibiotic discharge from liposomes at 4°C (—■—), room temperature (··▲··), and at 37°C (–●–). The results are expressed as the arithmetic mean of three samples ± S.E.M.

FIGURE 1


900,00
800,00
700,00
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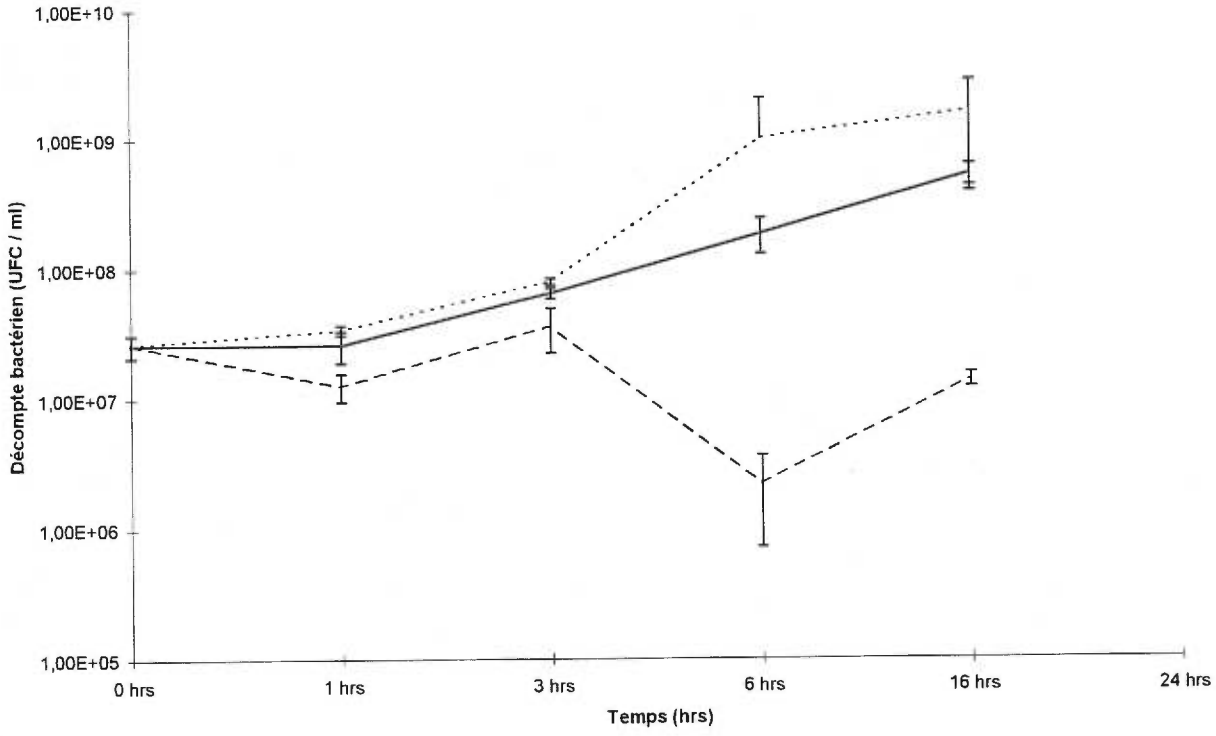
400,00
300,00
200,00
100,00
0,00
Tobramycin (µg)
0
0,66
1
2
14
21
28
time (days)

Figure 2. Killing curves of bacteria exposed to sub-MIC of free and encapsulated tobramycin respectively for: a) *P. aeruginosa*, 30 and 29.4 mg/L; b) *X. maltophilia*, 1.00 and 1.00 mg/L; c) *B. cepacia*, 3.00 and 2.95 mg/L; d) *E.coli*, 0.75 and 0.75 mg/L and e) *S. aureus*, 1.57 and 1.55 mg/L. The growth of bacteria was evaluated by cfu at 0, 1, 3, 6 and 16 h. The solid lines (—) on each graph represent bacteria exposed to free tobramycin, the dashed lines (-----) represent bacteria exposed to PBS (controls) and the dotted lines (- - -) represent bacteria exposed to liposome-entrapped tobramycin. MICs of tobramycin for *P. aeruginosa*, *X. maltophilia*, *B. cepacia*, *E. coli* and *S. aureus* were found to be respectively of 60, 2, 5, 1.5 and 2 mg/L. Results are expressed as the arithmetic mean of the three samples ± S.E.M.

FIGURE 2 A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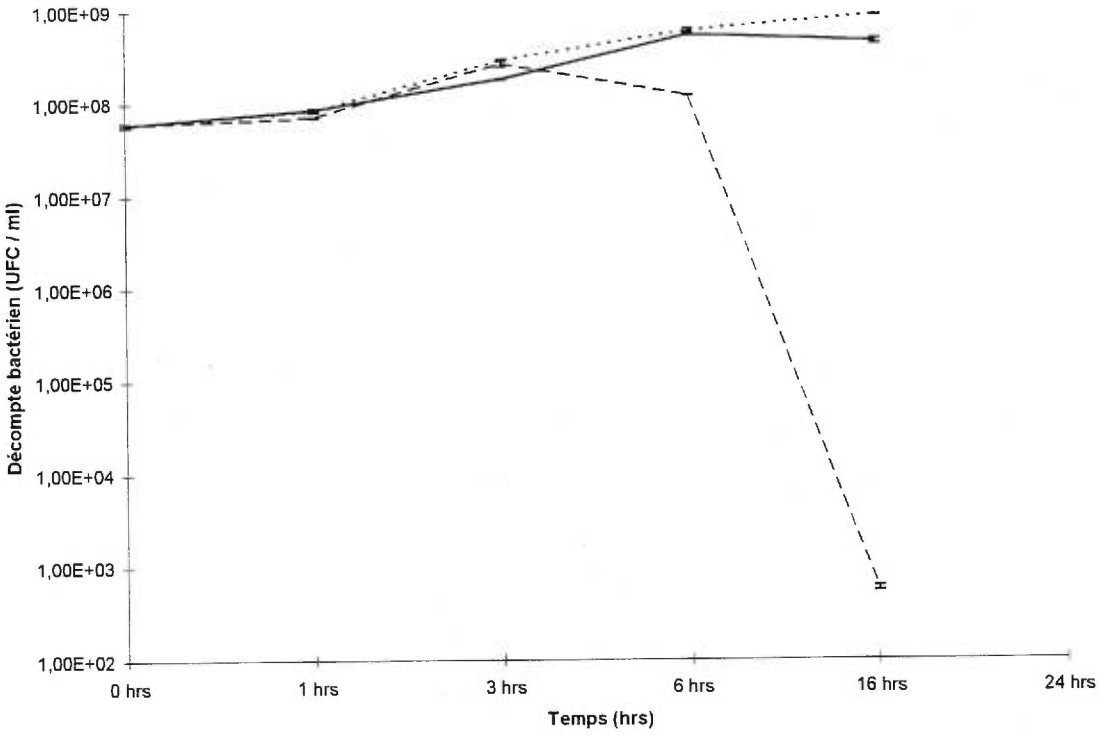
0 hrs
1 hrs
3 hrs
6 hrs
16 hrs
24 hrs
Temps (hrs)

FIGURE 2 B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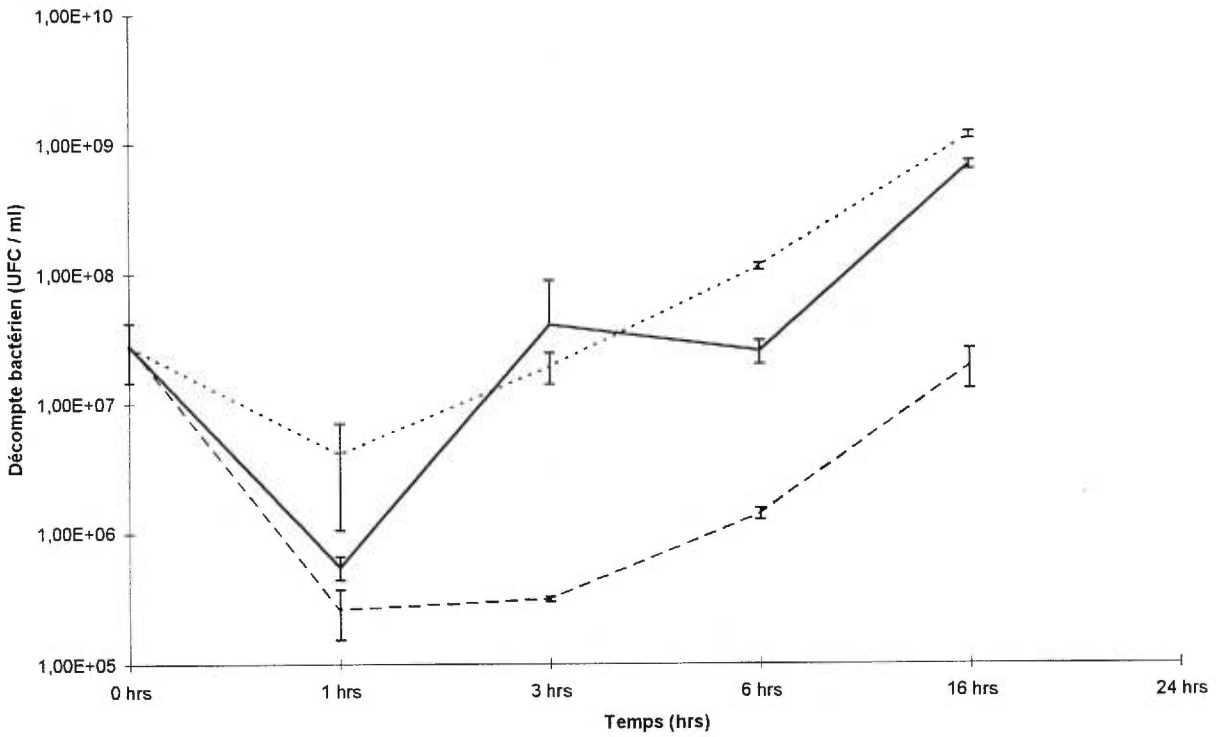
Décompte bactérien (UFC / ml)
0 hrs
1 hrs
3 hrs
6 hrs
16 hrs
24 hrs
Temps (hrs)

FIGURE 2 C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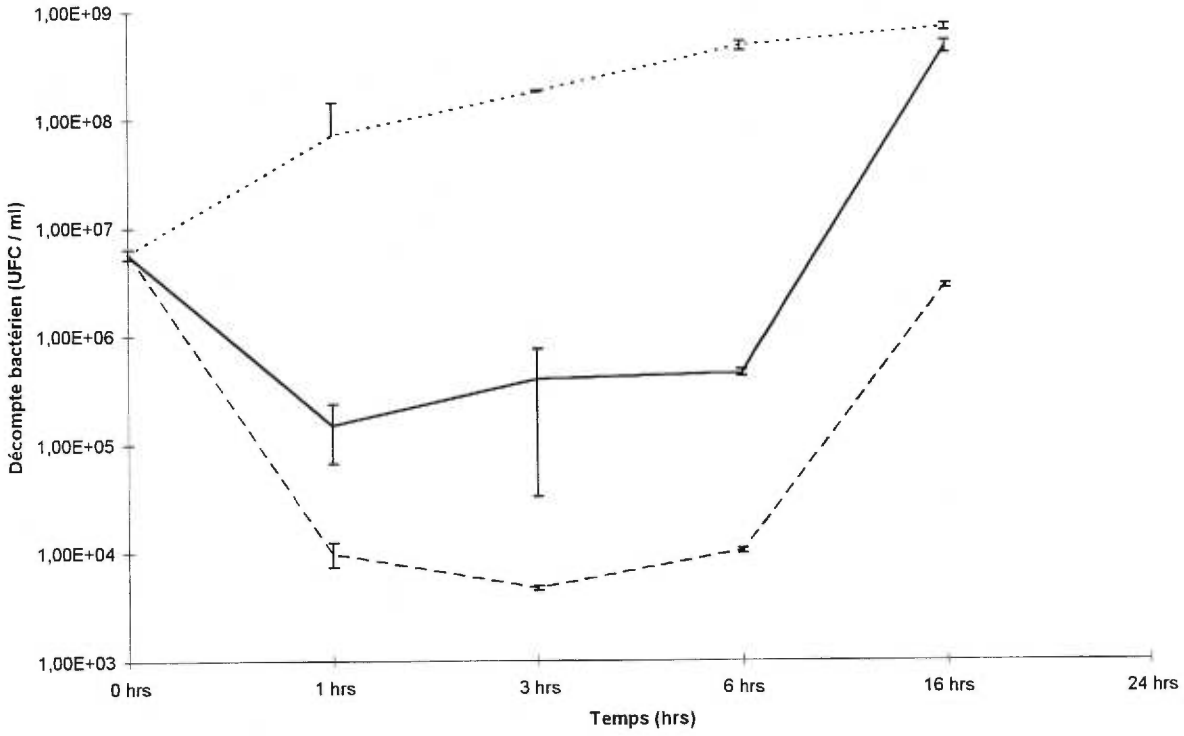
Décompte bactérien (UFC / ml)
0 hrs
1 hrs
3 hrs
6 hrs
16 hrs
24 hrs
Temps (hrs)

FIGURE 2 D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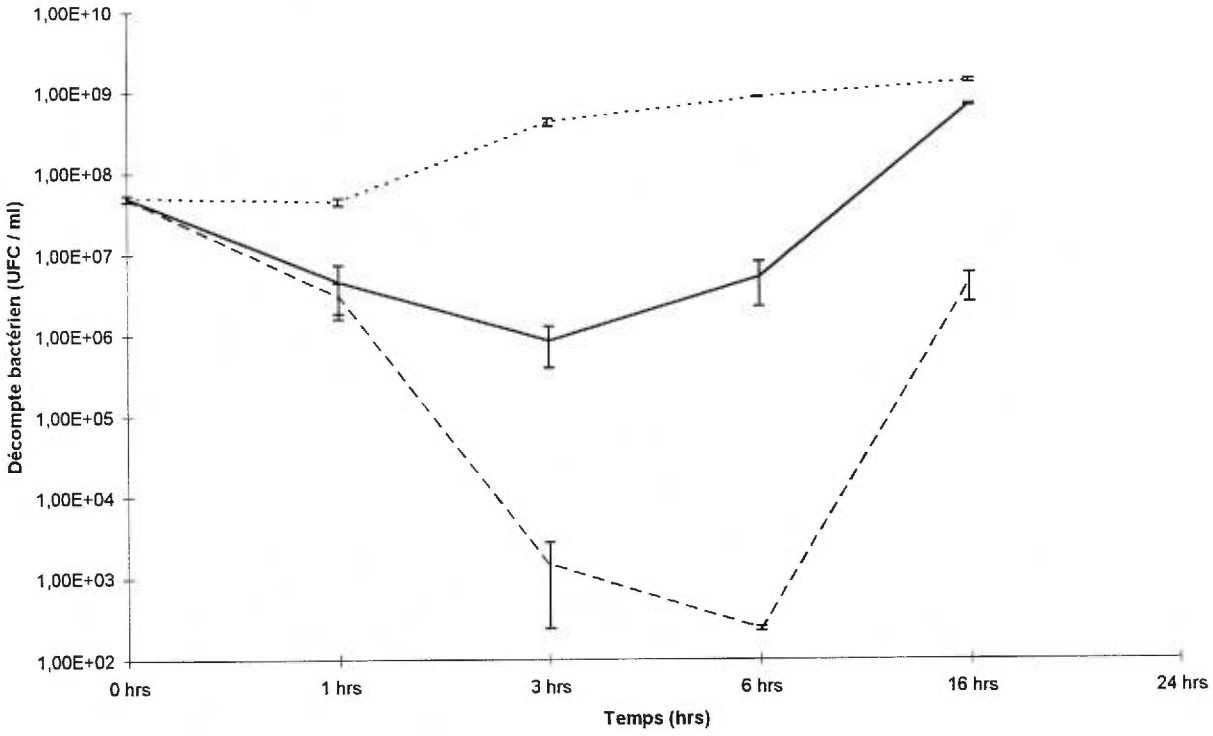
0 hrs
1 hrs
3 hrs
6 hrs
16 hrs
24 hrs
Temps (hrs)

FIGURE 2 E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Décompte bactérien (UFC / ml)
0 hrs
1 hrs
3 hrs
6 hrs
16 hrs
24 hrs
Temps (hrs)

CHAPITRE IV

IN VITRO BACTERICIDAL EVALUATION OF LOW PHASE TRANSITION TEMPERATURE LIPOSOMAL TOBRAMYCIN AS DRY POWDER PREPARATION AGAINST GRAM NEGATIVE AND GRAM POSITIVE BACTERIA

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Running heading: Bactericidal powder form liposome with sub - MIC antibiotic

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Summary

In previous studies, delivery of liquid preparation of encapsulated antibiotic in fluid liposomes has showed a marked improvement in the bactericidal activity against in-vitro and in-vivo extracellular infections. To examine the possibility to develop aerosol treatment using dehydrated liposome associated antibiotics to treat chronic pulmonary infections, lyophilized preparations of tobramycin and liposomal tobramycin were tested against cultures of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Escherichia coli* and *Staphylococcus aureus*. Bacterial colonies were enumerated 0, 1, 3, 6 and 16 h after the addition of the antibiotic. Sixteen hours post-treatment, the growth of *P. aeruginosa*, *S. maltophilia*, *B. cepacia* and *E. coli*, in the presence of sub-minimal inhibitory concentrations of tobramycin, was lowered respectively by 17, 40, 47 and 50 times in comparison with growth in the presence of free antibiotic. No improvement was observed against *S. aureus*. Results obtained in this study suggest that : 1) the dehydrated form of liposome antibiotic comprising low phase transition temperature phospholipids maintain the ability to increase penetration of antibiotic in gram negative bacterial cells; 2) the development of aerosolization methods to administer dehydrated liposomes associated with high concentrations of antibiotic could be a practical and efficient way to treat chronic pulmonary infections caused by resistant bacteria.

Introduction

Over the past 30 years, several treatments have been developed to improve survival in cystic fibrosis (CF) patients which resulted in increasing the number of children graduating to adult hood with the disease. Cystic fibrosis patients generally develop pulmonary infections with *Haemophilus influenzae* and *Staphylococcus aureus* followed by lung colonization with *Pseudomonas aeruginosa* and/or related strains like *Burkholderia cepacia* and *Stenotrophomonas maltophilia* [1-5]. Chronic pulmonary infections with *Pseudomonas* occur in 70-90% of patients with CF and is associated with high rates of morbidity and mortality. The most common anti-Pseudomonal treatment is a combination of an aminoglycoside and b-lactam antibiotics. In spite of the development of newer antimicrobial agents with less toxicity, tobramycin remains the drug of choice in the treatment of CF pulmonary infections [6]. Nevertheless whatever the antibiotic used, chronic pulmonary infections due to *Pseudomonas* in CF patients are rarely eradicated. Recently, it was demonstrated that tobramycin encapsulated in a fluid liposomal formulation succeed to eradicate chronic pulmonary infection caused by *P. aeruginosa* in an animal model [7]. Moreover in-vitro studies showed that sub-minimal inhibitory concentrations (sub-MIC) of fluid liposome encapsulated tobramycin reduced the growth of *P. aeruginosa, B. cepacia, S. maltophilia, Escherichia coli* and *S. aureus,* by factors of 84, 129,166, 10^5 and 10^4 times respectively comparatively to free tobramycin. In the aim of developing practical ways to administer fluid liposomes to humans, aerosolization of both liquid and dehydrated liposomes are considered. Studies on dehydrated liposomes is particularly attractive since dry powder inhaler systems are known to be less time consuming and

generally more effective per mg of drug than nebulized liquid drugs [8]. The aim of the present study was to verify if tobramycin contained in a fluid liposomal preparation used as a dry powder could maintain a high level of bactericidal efficiency against *P. aeruginosa*, *B. cepacia*, *S. maltophilia*, *E. coli* and *S. aureus* as previously demonstrated with the liquid form of the same liposomal formulation [9].

Material and Methods

Bacterial strains.

Clinical strains of *P. aeruginosa* (PA 429), S. *maltophilia* (XM 47), and *E. coli* (EC HB 101 NM 88) were isolated from the sputum of patients with cystic fibrosis (Sainte-Justine Hospital, Montréal, Québec, Canada). Clinical stains of *B. cepacia* (ID-28369) and *S. aureus* (LSQP 2499) were a generous gift from the Laboratoire de Santé Publique du Québec (LSPQ).. Microorganisms were stored at -70 °C in Brain Heart Infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 % glycerol.

Antimicrobial agents

Fluid liposome encapsulated tobramycin or control liposomes with phosphate buffered saline (PBS) were a generous gift from Theralipids Inc. (Theralipids inc, Pointe Claire, Québec, Canada). Tobramycin in an injectable solution is available under the trade mark Nebcin (Eli Lilly , Toronto, Ontario, Canada).

Preparations of liposomal tobramycin, free tobramycin, PBS and liposome PBS were furnished as dry powders under vacuum and kept at -70 °C until use. For each assay, precise amounts of tobramycin were added to a dry film of liposomes before lyophilization. The phospholipid content of each batch of liposomes was determined using the method of Fiske and Subbarow [10].

Antibiotic and suceptibility testing

Resistance to tobramycin was measured by broth microdilution assay in accordance with the procedures recommended by the National Commitee for Clinical Laboratory Standards [11]. Briefly, a bacterial inoculum of 50 ml corresponding to 5×10^5 colony forming units (cfu) / mL (0.02 O.D., 660 nm), were added to 50 ml of serial two-fold dilutions of tobramycin into microdilution wells (Sarstedt, Québec, Canada). The plates were incubated at 37 °C for 20 h. prior to the determination of MICs. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth. MICs were confirmed by E-Test (AB BIODISK, Sweden) following the manufacturer's recommendations. Briefly, after overnight growth on Muller Hilton (MH) plates at 37 °C under 5 % CO_2, colonies were resuspended in 0,9 % of saline to correspond to a 0,5 McFarland Standard density. This suspension was then triple-streaked on MH plates to insure confluent growth. The carrier strips containing the predefined tobramycin gradient were then applied to the inoculated MH plates and incubated for 24 hours at 37 °C. At the end of the incubation period, the elliptical zone of inhibition around the strip gave the MIC. The antibiotic concentration used in those time kill curves was calculated by using 50 % or less of the MIC values obtained by the E-Test determination. MICs were determined three times in triplicate.

Experimental desing

In-vitro killing curves were performed as previously described [11-13] with modifications. Studies were performed in the presence of sub-MIC concentrations of free tobramycin or liposome associated tobramycin and controls made of liposome associated PBS or PBS alone. Briefly, volumes of 250 mL of Proteose Peptone Broths no 2 (Difco Laboratories, Detroit, USA) were inoculated with a 17 h pre-culture of each bacteria to give a stock preparation containing a mid-logarithmic growing final inoculum of 5×10^5 cfu/mL as evaluated initially by O.D. at 660 nm. This value allowed control growth to reach a density of 10^8 to 10^{10} cfu/mL. To confirm bacterial growth, culture dilutions were plated in triplicate and cfu were enumerated.

All the killing tests were performed in triplicate as follows: 30 mL of the cultures described above were added directely to vial containing dry powder doses of free tobramycin, liposome associated tobramycin, liposome PBS and PBS. Quantities of tobramycin contained in the dry powder preparations have been added before lyophilization and were exactly the same for the liposomes and for the free drug. Cultures of *P. aeruginosa, S. maltophilia, B. cepacia, E. coli* and *S. aureus* received respectively 30.00, 1.00, 3.00, 1.50 and 1.00 mg of tobramycin per mL of culture. These values were two times lower than the respective MIC of each bacterial strain. The cultures were then incubated on a shaker at 37 °C for 16 h. Samples (1 mL) were collected at regular intervals and serially diluted in sterile PBS. Viable colony-count determinations were done from three 100 ml samples from each dilution (final dilution of 10^{-8}) and streaked by platting on Proteose Peptone no. 2 agar (Difco

Laboratories) to insure colony isolation. After an overnight incubation at 37°C in 5 % CO_2, the colonies were counted and averaged. In-vitro killing curves were performed in triplicate in three independant experiments.

Data analysis

Bacterial counts were expressed as the mean +/- standard error from the mean obtained from at least three plates per dilution. All computer analysis were performed with a linear regression using Microsoft EXCEL version 5.0.

Results

Susceptibility testing

Comparative MIC determinations using broth microdilution assay (BMDA) following NCCLS recommendations and E-Test are presented in table 1. The two tests gave identical MIC values for the strain # 429 of *P. aeruginosa* that presents a very strong antibiotic resistance corresponding to 60 mg/L. Nevertheless, for low antibiotic resistance near bordeline values (MIC £ 8 mg/L), the BMDA method showed greater MIC values (differences of 1 to 6 mg/L) than the E-Test (Table 1).

Comparative efficiency of dehydrated liposome associated-tobramycin and free tobramycin.

At the beginning of the bactericidal study, the mean concentrations of *P. aeruginosa, S. maltophilia, B. cepacia, E. coli* and *S. aureus* were respectively of 2.83×10^7, 2.48×10^8, 5.41×10^7, 5.62×10^7 and 3.89×10^7 cfu per mL. The number of cfu for the different bacterial strains after cultures of 1, 3, 6 and, 16 h with free tobramycin, liposomal tobramycin and controls are presented in Fig. 1. As indicated in materials and methods, the doses of liposomal tobramycin and free tobramycin were lower than the MIC being eiher 30, 1, 3, 1.5 and 1 mg/L respectively for *P. aeruginosa, S. maltophilia, B. cepacia, E. coli* and *S. aureus.* Killing curves of bacteria were evaluated by enumeration of cfu on agar plates (Fig.1a-e). Controls made of liposomal PBS were not included in the figures because of the superimposition of these results with the PBS controls. As an exemple, the 16 hrs values of these controls were $2,6 \times 10^8$, $6,35 \times 10^9$, 2.53×10^{10}, 2.25×10^{10} and $6,37 \times 10^9$ cfu/mL comparatively to 3.35×10^8, 6.48×10^9, 2.52×10^{10}, 2.23×10^{10} and 1.26×10^{10} cfu/mL for PBS control values.

Six hours after the addition of liposome associated-tobramycin to *S. maltophilia, B. cepacia* and *E. coli* cultures, the reduction of growth was respectively of 2.5, 13.5, and 20.3 times comparatively to the same cultures treated with free antibiotic (Figure 1b, c,d); sixteen hours after the addition of liposomal antibiotics to the same cultures, the reduction in growth reached respectivement 40, 47 and, 50 times the values obtained with free antibiotic ($P < 0.001$). *P. aeruginosa* cultures showed a reduction in growth corresponding to 17 times compared to that of cultures treated with free antibiotic only after 16 h. (Figure 1a). Culture of *S. aureus,* the only gram positive strain tested, did not show any significant difference between numbers of cfu following the

addition of liposomal tobramycin comparatively to free antibiotic. In PBS control cultures, bacterial growth reached approximately 10^8 - 10^{10} cfu/mL depending of the strain (Figure 1a-e).

Discussion

Many years of antibiotic usage have selected out drug-resistant strains that utilize several mechanisms to develop resistance. These include degradation or inactivation of the drug by enzymatic modifications, alteration of the drug target and less specific mechanisms such as the permeability barrier and the associated active efflux in which access of the unaltered agent to the target is prevented[14]. The principle of the efflux pumps that seem to be an important element in the *P. aeruginosa* resistance to antibiotics and in other related bacteria like *B. cepacia* and *S. maltophilia* appears to be a common phenomenon in other resistant pathogens [15]. Indeed, it was demonstrated that many other groups of bacteria are also able to extrude drugs from their cytoplasm. For example, a system of efflux pumps has been identified in *Bacillus subtilis* [16], *E. coli* [17] and *S. aureus* [15, 18]. Another important cause of bacterial resistance to antibiotics that is frequently underestimated, seems to be an adaptive unstable permeability defect that has been well documented in *P. aeruginosa* following exposure to an aminoglycoside [19, 20]. This reversible refractoriness to the bactericidal action of an antibiotic following first exposure appears to relate to a reversible down-regulation of drug uptake into the bacteria, especially during the period of accelerated energy-dependant drug transport [21]. This phenomenon has been first well documented *in vitro* and in a mouse model of *P. aeruginosa* infections following exposure to an aminoglycoside [19, 21-23]. Recently, adaptive resistance to the bactericidal action

of tobramycin was identified in *P. aeruginosa* in the sputum of 7/7 patients with CF analyzed soon after an inhalation dose [24]. Many factors demonstrate that such resistance is not due to selection of mutant strains: reversibility, the constant nature of the MICs for bacteria obtained before, during, and after the period of resistance and strain conservation as demonstrated by sensitive genomic fingerprinting techniques [24] are proof of this.

The use of liposomes as drug delivery system to improve drug therapy traditionally relies on three rationales : pharmacokinetic and biodistribution modifications, administration and / or site-directed delivery and reduced toxicity of chemotherapy. Current liposomal formulations include cholesterol and/or phospholipids which have a high phase transition temperature (Tc) which makes the liposomes rigid and strong. Experimental models of infections in which antimicrobial agents encapsulated in rigid liposomes have been successful in both in-vivo and in-vitro studies all involved infection of cells of the mononuclear phagocyte system (MPS) by facultative or obligate intracellular pathogens [25,26]. In these studies, uptake of rigid liposomes by phagocytes was essential to obtain a superior therapeutic effect. On the other hand, the delivery of antibiotic encapsulated in fluid negatively charged liposomes made of phospholipids with low T_C showed a marked improvement of bactericidal activity against in-vitro and in-vivo extracellular infections even when initiated with resistant strains [7, 9, 27]. The superior bactericidal effect of fluid liposome encapsulated antibiotic results from its ability to directly interact with the bacterial membrane allowing increase penetration of drugs in the bacterial cell (unpublished results).

In view of a possible clinical development of aerosol treatment of chronic pulmonary infections

with liposome associated antibiotics, liposomes in dry powder form may be an attractive solution for many reasons: stability of the drug, simplicity and rapidity of the administration and general greater efficiency per mg of drug than nebulized liquid drugs [8]. In this study, we demonstrate that sub-MIC concentrations of low T_C liposomal tobramycin added as a dry powder preparation to cultures, after 16 h., reduced the number of cfu of *P. aeruginosa, S. maltophilia, B. cepacia* and *E. coli*, by factors of 17, 40, 47 and 50 respectively comparatively to the same quantity of free powder antibiotic. If we compare these latter results obtained against the same gram negative strains, to those obtained with liquid liposomes, they seem to be less effective : indeed, the addition of liquid liposomes to cultures lowered by 84 to 10^5 times the number of cfu in comparison with free antibiotic [9]. Moreover, the powder form of liposome associated tobramycin against *S. aureus* did not show any significant improvement of bactericidal activity comparatively to free tobramycin whereas a difference of 10^4 was observed with the liquid liposomal preparation [9]. It is difficult to explain why the growth of the gram positive strain was not lowered by the dry powder preparation of liposomal tobramycin. It maybe that the wall of gram positive bacteria is less receptive to liposomes rehydrated in non optimal conditions. The greater efficiency of the liquid form of liposomes compared to the dehydrated form against gram negative bacteria may be explained by different factors. The usual preparation of liquid liposomes comprises a rehydration step followed by washes to eliminate unencapsulated antibiotic and consequently only encapsulated tobramycin is used in the assays. To prepare the liposomes in dry powder form, a precise quantity of tobramycin is added to phospholipids before lyophilization and because liposomes are used as a powder, unencapsulated tobramycin cannot be eliminated

and must be considered in the total therapeutic dose. Studies, performed to determine the quantity of antibiotic effectively encapsulated in liposomes following direct incorporation of powdered liposomal tobramycin in medium culture at 37° C, have shown a mean of 73.5% of encapsulation for doses of 600 mg of tobramycin (data not shown). The low doses of tobramycin used as dry powder in the present study did not allow us to verify with certainty the percentage of encapsulation. Nevertheless, preliminary studies suggest that these values may be as low as 10% (data not shown). It is important to mention that clinical treatments will require doses of antibiotic that will be sufficient to avoid such problems.

In conclusion, if we consider the following factors: the advantage of inhalers versus nebulizers [8], the feasibility to aerosolize dehydrated lipoomes 28, the bactericidal efficacy of the dehydrated liposomal tobramycin, this study suggests that antibiotherapy based on the administration of such liposomal preparation could be a well adapted answer to the resistance problem of gram negative bacteria in CF and in other patients presenting chronic pulmonary infections. Further studies that examine the therapeutic values of aerosolized dry powder preparation of liposomal tobramycin in the management of experimental pseudomonas pneumonia in animals are in progress.

Acknowledgement

This study was supported by a grant from the Canadian Cystic Fibrosis Foundation.

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Tableau 1

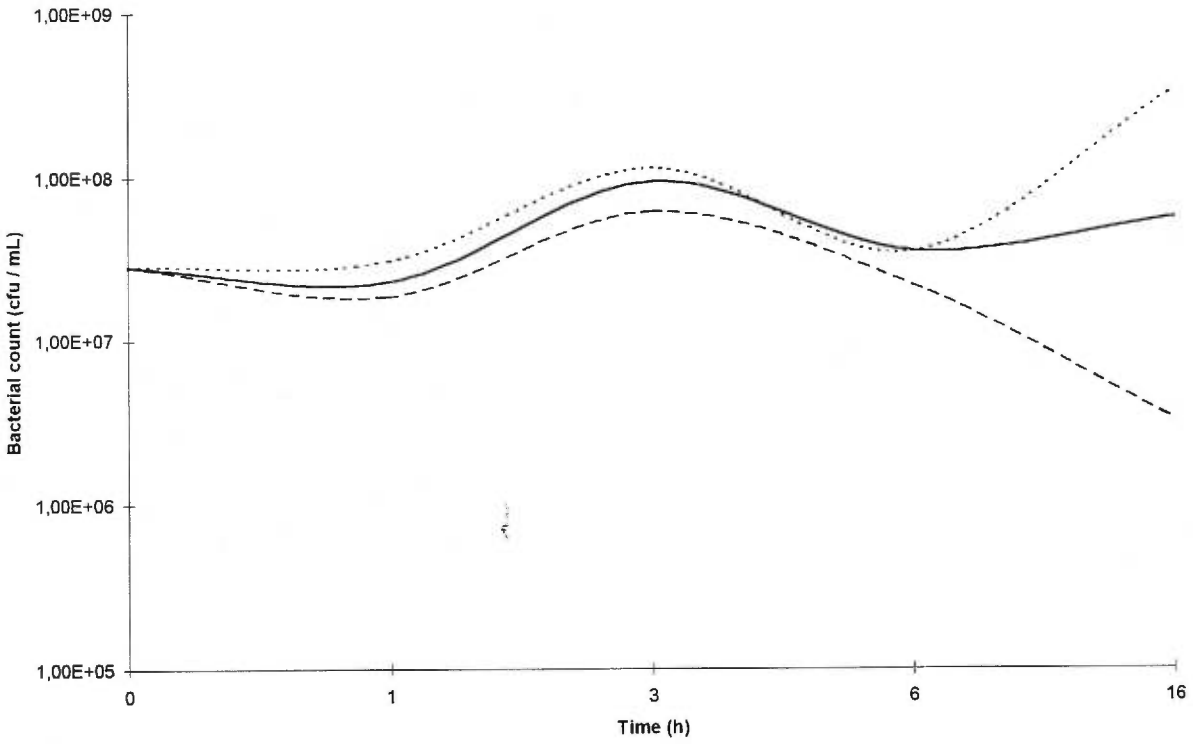
Table 1

Strain	E-Test MIC method (mg/L)	BMDA MIC (mg/L)
Pseudomonas aeruginosa # 429	60	60
Stenotrophomonas Maltophilia	2	6
Burkholderia cepacia	5	6
Escherichia coli	1,5	5
Staphylococcus aureus	2	8

Table 1. Comparative tobramycin MIC evaluation between E-Test and the NCCLS broth microdillution method (BMDA) for strains of *P. aeruginosa, S. maltophilia, B.cepacia, E. coli* and *S. aureus.*

Figure 1. Killing curves of bacteria exposed to sub-MIC of free and encapsulated tobramycin respectively for: a) *P. aeruginosa*, 30.00 mg/L; b) *S. maltophilia*, 1.00 mg/L; c) *B. cepacia*, 3.00 mg/L; d) *E.coli*, 1.50 mg/L and e) *S. aureus*, 1.00 mg/L. The growth of bacteria was evaluated by cfu at 0, 1, 3, 6 and 16 h. — bacteria exposed to free tobramycin; ----- bacteria exposed to PBS (controls); - - - bacteria exposed to low phase transition temperature liposomal tobramycin. MICs of tobramycin for *P. aeruginosa*, *S. maltophilia*, *B. cepacia*, *E. coli* and *S. aureus* were found by E-Test MIC method to be 60, 2, 5, 1.5 and 2 mg/L respectively. Results are expressed as the arithmetic mean of the three samples ± S.E.M.

Figure 1 A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1,00E+07
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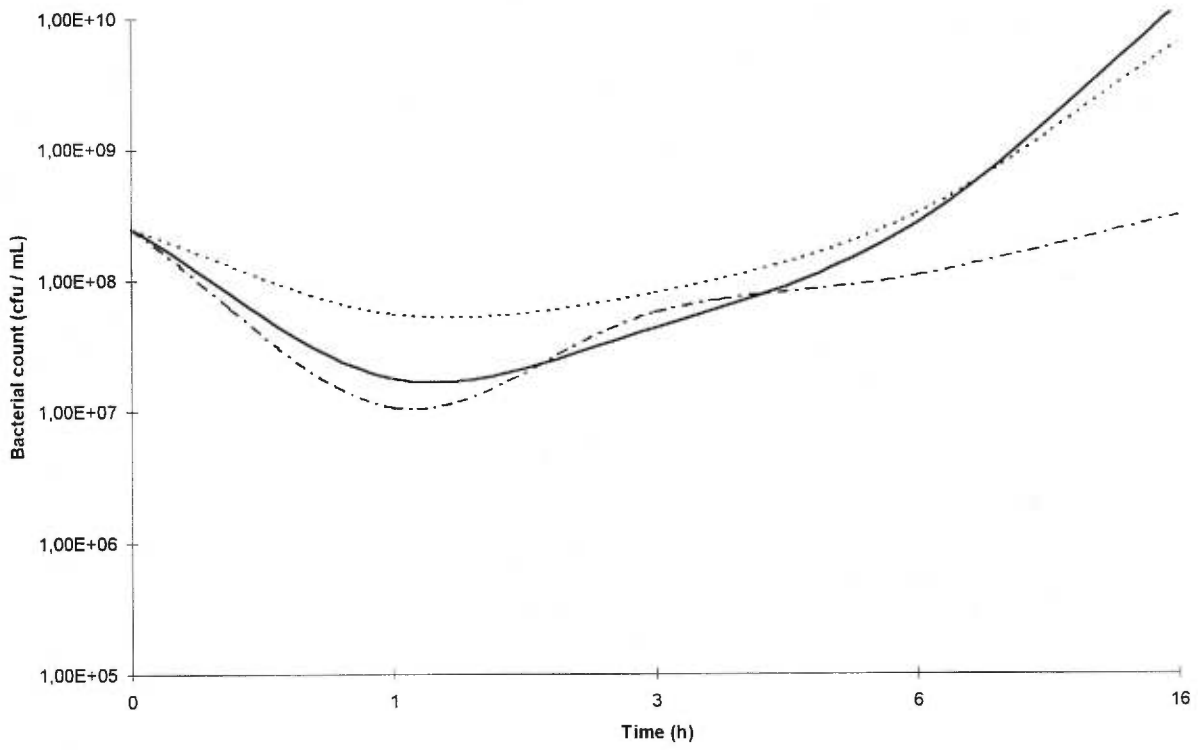
Bacterial count (cfu / mL)
0
1
3
6
16
Time (h)

Figure 1 B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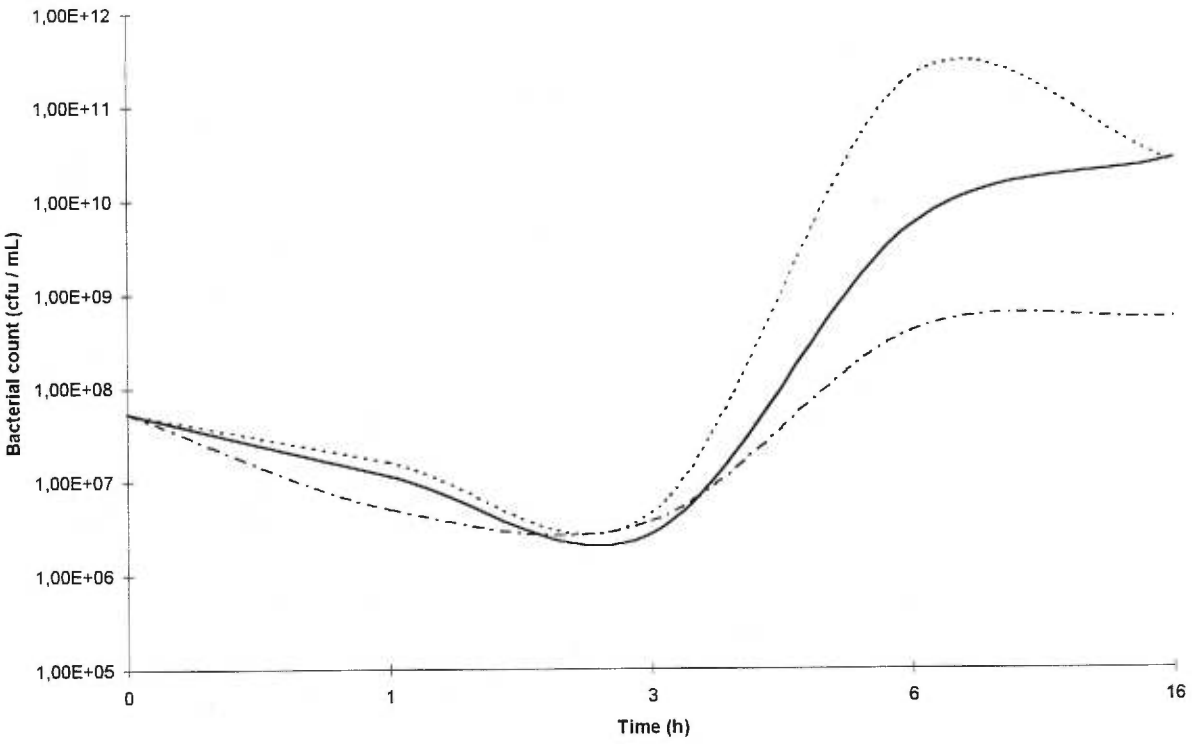
Bacterial count (cfu / mL)
0
1
3
6
16
Time (h)

Figure 1 C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1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
Bacterial count (cfu / mL)
0
1
3
6
16
Time (h)

Figure 1 D

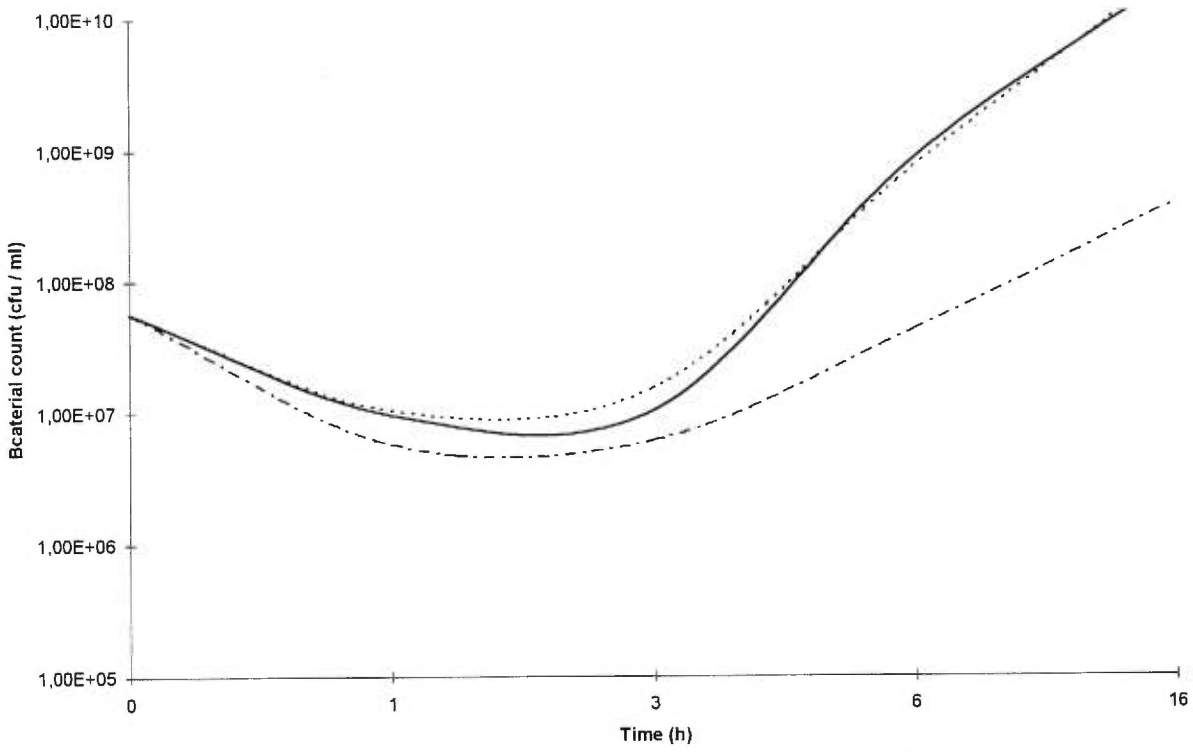
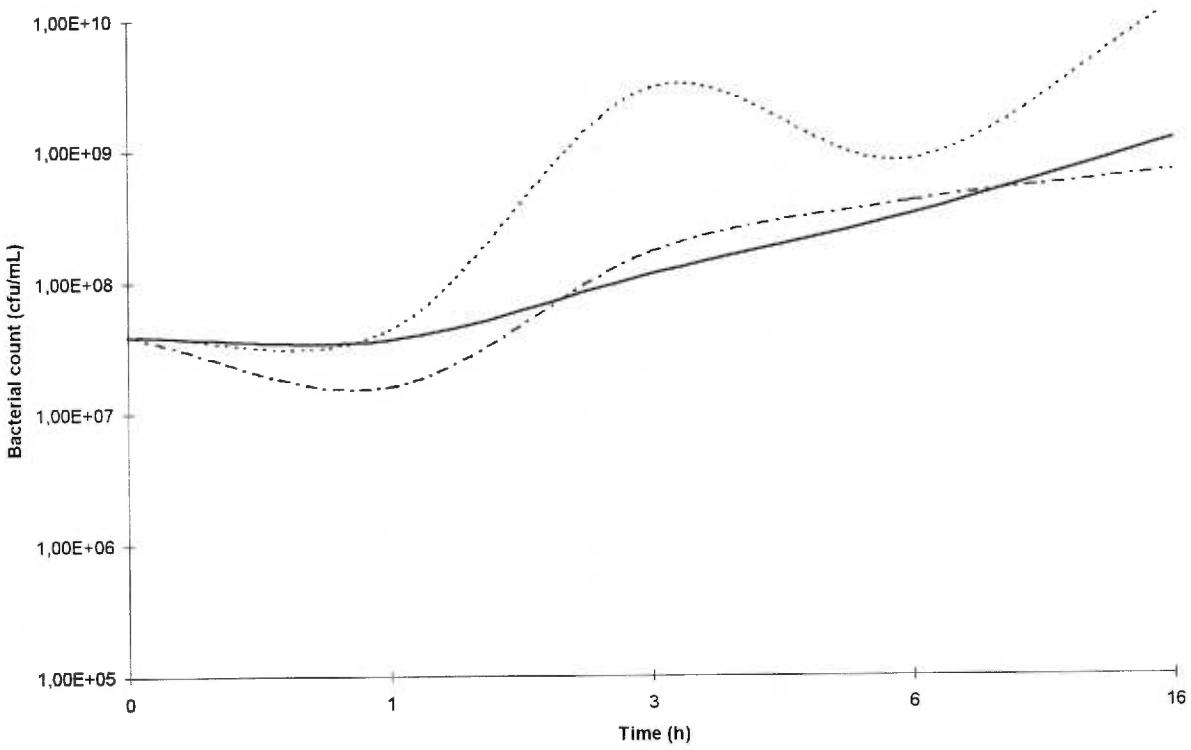

1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
Bcaterial count (cfu / ml)
0
1
3
6
16
Time (h)

Figure 1 E


1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
Bacterial count (cfu/mL)
0
1
3
6
16
Time (h)

CHAPITRE V

AEROSOLIZATION OF LOW PHASE TRANSITION TEMPERATURE LIPOSOME-TOBRAMYCIN AS DRY POWDER IN AN ANIMAL MODEL OF CHRONIC PULMONARY INFECTION CAUSED BY *PSEUDOMONAS AERUGINOSA*

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Abstract.

Eradication of mucoid *Pseudomonas aeruginosa* in an animal model of chronic pulmonary infection has been previously demonstrated following the intratracheal administration of liquid form of fluid liposome-encapsulated tobramycin (Beaulac et al., 1996. Antimicrob. Agents Chemother **40** : 665-669). In the present work, the same fluid liposomal formulation was administered as dry powder aerosols to the animal model of chronic pulmonary in view of a possible clinical development in cystic fibrosis patients. Chronic infection was established by intratracheal administration of 10^5 cfu of a mucoid variant of *P. aeruginosa* PA 508 prepared in agar beads. Sixteen hours after one aerosol treatment, the cfu counts performed from lungs (pair) treated with liposome-tobramycin was of 4.31×10^5 comparatively to 3.02×10^8 and 1.32×10^8 respectively in lung controls and in lungs treated with free antibiotic. Considering the number of treatments and the quantity of liposome-tobramycin administered in animals, results suggest that aerosolized fluid liposome-tobramycin used as dry powder could be as effective as the liquid form of fluid liposome-tobramycin to treat chronic pulmonary infections caused by *Pseudomonas*.

Introduction.

Cystic fibrosis (CF) is a genetic disease where the basic defect is due to abnormality of chloride ion transport across epithelial cells lining ductal systems (43). This pathology affects many body systems, however, almost total mortality can be attributed to progressive lung damage with the mean age of death occuring in the third decade of life (2, 16, 17, 20, 23). Lung damage is due to a combination of progressive pulmonary sepsis and exuberant self-damaging host responses (15, 24, 30, 32). The aim of CF care is based on aggressive use of oral, intravenous and nebulized antibiotics to slow down progression of lung disease . Those treatements are palliative rather than curative since despite the use of aggressive antibiotic therapy, *Pseudomonas aeruginosa*, the enemy number one in CF patients, is rarely eradicated at present (22, 38, 41). Difficulties to eradicate infections caused by *P. aeruginosa* and other related bacteria like *Burkholderia cepacia* and *Xanthomonas maltophilia* are due in large part to their high resistance to antibiotics. *Pseudomonas* have an outer membrane with a low level of permeability combined to a multiple drug efflux transporter (39) that makes it intrinsically resistant to a wide variety of commonly used antibiotics including the majority of b-lactams, tetracyclines, chloramphenicols and fluoroquinolones (1, 27, 35, 45). Antibiotic resistance of *Pseudomonas* may be mediated not only by an increase of their impermeability barriers but also by other mechanisms that can act synergistically like production of antibiotic-inactivating enzymes (25, 28, 29) and/or alteration of antimicrobial targets. Among the above-mentioned mechanisms, low outer-membrane permeability and drug efflux pumps seem to play a predominant role (11, 18, 35). Another important cause of bacterial resistance to antibiotics that is

frequently underestimated, seems to be an adaptive unstable permeability defect that has been well documented in *P. aeruginosa* following exposure to an aminoglycoside (4, 19). This reversible refractoriness to the bactericidal action of an antibiotic following first exposure appears to relate to reversible down-regulation of drug uptake into the bacteria, especially during the period of accelerated energy-dependant drug transport (14). Recently, adaptive resistance to the bactericidal action of tobramycin was identified in *P. aeruginosa* in the sputum of 7/7 patients with CF analyzed soon after an inhalation dose (5). Many factors demonstrate that such resistance is not due to selection of mutant strains: reversibility, the constant nature of the MICs for bacteria obtained before, during, and after the period of resistance and, strain conservation demonstrated by sensitive genomic fingerprinting techniques (5). Biofilm formation by mucoid variants of *P. aeruginosa* in the lungs of CF patients may be also an important cause of infection persistence (12, 26, 33).

Liposomes, a drug delivery system, could be one way of improving the effectiveness of antibiotics in modifying pharmacokinetics, biodistribution and interaction of encapsulated drug (9,13, 34, 40, 42). The use of drugs encapsulated in conventional liposomes presenting high phase transition temperature (T_C), which makes the liposomes rigid and stable, have shown effectiveness in reducing drug toxicity and infection caused by facultative or obligate intracellular pathogens involving cells of the mononuclear phagocyte system (31, 37). However, rigid liposomes have generally low bactericidal activity against extracellular bacteria since uptake of rigid liposomes by phagocytes seems essential to obtain a superior therapeutic effect (3, 21).

During the last years, our laboratory has investigated the potential to deliver antibiotics to extracellular infections using fluid liposomes made of synthetic phospholipids and characterized by their low phase transition temperature (T_c) and a net negative charge. These fluid liposomes that can directly interact with the bacterial walls succeed to eradicate mucoid *P. aeruginosa* in an animal model of chronic pulmonary infection whereas antibiotic encapsulated in rigid liposomal preparations or free antibiotic were not bactericidal (9, 36). Afterwards, it was demonstrated in *in vitro* studies that sub-minimal inhibitory concentrations (sub-MIC) of liposome-encapsulated tobramycin succeed to reduce the growth (number of cfu) of *P. aeruginosa, Burkholderia cepacia, Xanthomonas maltophilia, Escherichia coli* and *Staphylococcus aureus,* 84 to 10^5 times more than the same quantities of free antibiotic. Taking into consideration that the preparation of fluid liposomes requires a lyophilization step and the practical advantages of powder form of drugs for aerosolization, bactericidal activity of fluid liposomes in powder form was first tested in *in vitro* studies. This study showed that fluid liposomes associated-antibiotic administered as dry powder maintained a higher bactericidal activity than free antibiotic (17 to 50 times superior) against *P. aeruginosa, Burkholderia cepacia, Xanthomonas maltophilia and, Escherichia coli* (8). The aim of this present work was to test the bactericidal efficacy of aerosolized low T_C liposome- tobramycin administered as dry powder in an animal model of chronic pulmonary infection caused by a mucoid strain of *P. aeruginosa*.

Materials and methods.

Bacterial strain. A clinical isolated of *P. aeruginosa* , PA 508, was used throughout this study. PA 508 is a stable mucoid strain isolated from the sputum of a CF patient (Sainte Justine Hospital, Montreal, Québec, Canada). The minimal inhibitory concentration (MIC) of tobramycin against PA 508 is 1 mg/ml. Bacteria were stored at - 70 °C in brain heart infusion broth supplemented with 10 % glycerol. For the experimentation, a 17-h culture of this organism in Proteose Peptone broth (Difco Laboratories, Detroit, Mich.) was used.

Liposomes. Tobramycin (Eli Lilly , Toronto, Ontario, Canada) and PBS (control formulation) encapsulated in fluid liposomes were a generous gift of Theralipids inc (Theralipids inc, Pointe Claire, Québec, Canada). The quantification of tobramycin in liposomes was performed by high performance liquid chromatography (HPLC) as described below. Doses of encapsulated tobramycin, free tobramycin, encapsulated PBS or free PBS were placed into individual vials, lyophilized at 4 °C and sealed under vacuum (Virtis Genesis 12 EL Console freeze dryer; Canberra Parkard, Toronto, Ontario, Canada). Vials were kept at - 70 °C until use. To make certain that all doses were similar to others in terms of phospholipid and tobramycin quantities, powder preparations from ten vials were dissolved individually into 1 ml of sterile PBS. Secondly, to control the doses administered from the aerosolizer (microsprayer) (Penn-Century, Philadelphis, PA), powder preparations from ten vials were placed individually into the

aerosolizer and dispensed (5 shots) into 1 ml of sterile PBS. All the test samples were centrifuged (5000 g x 20 min), supernatants were recolted and each pellet was resuspended in 1 ml of PBS. Afterwards, 100 ml samples of total liposomal solutions, supernatants and resuspended pellets were submited to HPLC analysis for tobramycin determination as described below. Two hundred microliter samples of the same preparations were also submited to phospholipid determinations using a modified method of Fiske and Subbarow (6). All experiments were performed in triplicate.

Tobramycin quantification. The procedure for the determination of tobramycin in liposomes was based on derivatization of tobramycin with 1-fluoro 2,4-dinitrobenzene (FDNB) as previously described (9). Briefly, a volume of 170 ml of supernatant from a tobramycin methanol (MeOH) extraction solution was transfered in a vial containing 90 ml of FDNB-MeOH solution, 70 ml of sodium borate (0.1 M) and 670 ml of MeOH. Vials were sealed, vortexed and heated for 30 min at 82 °C and then cooled at room temperature. The limit of detectability for tobramycin in PBS and in lung and kidney tissues were 0,2 mg/ml, 0,3 mg/mg, and 0,5 mg/mg respectively. Correlation coefficients of 0,99% were observed for the chromatographic peak areas and the tobramycin concentrations between 0,2 and 125 mg/ml of PBS, between 0,3 and 125 mg/mg of lung tissue, and 0,5 and 125 mg/mg of kidney tissue samples. In PBS assays, the coefficients of variation at concentration of tobramycin from 0,2 to 125 mg/ml ranged between 0,6 and 1,2 %. For lung and kidney samples, the coefficients of variation at concentrations from 0,3 and 0,5 to 125 mg/mg ranged between 0,3 and 7,6 % and 1,7 % and 7,4 %, respectively. Three sets of five

quality control PBS samples at tobramycin concentrations of 3,9 and 125 mg/ml were analyzed to determine the intraday and interday variabilities of the assay; the coefficients of variation were of 0,01 and 0,1 %, respectively, for intraday measurements and 0,1 and 1,2 % for interday measurements. The samples were also analyzed by microbiological assay as described previously (9) to control the maintenance of the antibiotic activity. The limits of detection for tobramycin in PBS and tissues were 3,9 mg/ml and 7,8 mg/ml respectively. The correlation coefficients in PBS and tissues were 0,97 and 0,99 while coefficients of variation for intraday and interday measurements were £ 10 %.

Experimental design. One hundred and twenty six adult Sprague-Deawley rats weighing between 175 to 225 g (Charles River, Saint Constant, Québec, Canada) were used for this study. Animals were housed in groups of three and allowed free access to food and water. Chronic pulmonary infection caused *by P. aeruginosa* PA 508 was induced as described previously (36) with slight modifications. Briefly, the bacterial strain was grown overnight at 37 °C in Proteose Peptone broth (Difco Laboratories, Detroit, Mich.). Bacteria were then incorporated into agar beads and a suspension containing 5 X 10^5 CFU / 100 ml was administered intratracheally to anesthetized animals. Three days after the inoculation of coated bacteria, rats were anesthetized with a mixture of 70 mg of ketamine hydrochloride per kg of body weight (CDMV, St Hyacinthe, Québec, Canada) and 7 mg of xylazine (CDMV, St Hyacinthe, Québec, Canada) per kg by intramuscular injection. The rats were placed on a support in an upright position (45°) to

facilitate intubation with an intratracheal catheter possessing a polished stylet (20-25 mm depth depend of trachea size). The stylet was then withdrawn, and the intubation was confirmed by the formation of water condensation on a cold mirror. A delivery device for intratracheal administration of dry powder, the Penn Century intratracheal aerosolizer (microsprayer) (Penn-Century, Philadelphia, PA) was introduced throught the catheter to the bifurcation of the trachea. This microsprayer consist of a 5 ml syringe combined with a sub-miniaturized nozzel mounted on the tip of a 3 in, 18-gauge stainless steel tube. When the syringe plunger is depressed, the powder is forced at high pressure into a small mixing chamber at the end of the steel tube imparting considerable momentum to the powder. Dry doses of 600 mg of free tobramycin or combined to dry liposomes or dry liposomes alone were administered to rats. An additionnal group of untreated rats was also included in the study. At several times (0, 1, 3, 6 and 16 h) after treatment, animals were anesthetized, exsanguinated and killed. Kidneys and lungs were removed aseptically and homogenized in cold sterile PBS (40% wt/vol) for 30 s with a polytron Homogenizer (Polytron: Kinematica, Lucerne, Switzerland). A sample of 500 ml of homogenized lungs were immediately used for serial dilutions (10 fold) in PBS and appropriated dilutions were plated and cultured in triplicate on Proteose Peptone no. 2 agar (Difco Laboratories, Detroit, Mich.) in triplicate. After an overnight incubation, the number of CFU was determined. *P aeruginosa* colonies were also tested on C-390 medium agar to confirm identification. Samples (100 ml) of serum and homogenized tissues were conserved at - 70 °C in a methanol extraction solution before HPLC analysis. The remainder of the tissue homogenates was conserved at - 70 °C.

Data analysis. Bacterial counts were expressed as mean +/- standard error of the means obtained from at least three plates per dilution. All computer analysis were perfomed with a linear regression using Microsoft EXCEL version 5.0

Results.

HPLC analysis of 10 samples containing liposome-tobramycin and 10 samples containing free tobramycin in dry powder form indicated the presence of quantities of tobramycin corresponding to respectively 599.3 ± 8.98 and 595.7 ± 10.72 mg of antibiotic per vial. The coefficient of variation between free tobramycin samples was £ 0.6% whereas it was £ 1.8 %. for liposome-tobramycin. HPLC analysis of samples to measure quantities of antibiotic leaving the microspayer and dissolved in PBS reached values of 343.8 mg ± 74.4 (57.3%) for encapsulated tobramycin and 577.0 mg ± 14.76 (96.17%) for free antibiotic.

One hundred twenty six rats weighing 175 to 225 g were used in this study. The animals were divided into the following five groups: group 1 (n = 30), infected animals treated with free tobramycin; group 2 (n = 30), infected animals treated with liposome-tobramycin; group 3 (n = 30), infected animals treated with liposome-PBS; group 4 (n = 30), infected animal treated with PBS; group 5 (n = 3), infected animals without any treatment.

P. aeruginosa colony counts performed from dilutions of homogenized lung tissues made at time 0, indicated a mean cfu of 1.44×10^6 in all rats included in the study (Fig. 1). One , three and six hours after the treatment, no significant differences in the cfu counts were observed between the different groups. Sixteen hours after the treatment, the cfu counts performed from lungs treated with liposome-tobramycin were of 4.31×10^5 whereas those from lungs treated with liposome controls or with free

antibiotic reached values respectively of 3.02 x 10^8 and 1.32 x 10^8 cfu per pair of lungs (Fig. 1). Consequently, a cfu lowering of about 300 times was observed following the treatment with liposome-tobramycin comparatively to the use of free tobramycin. No significant difference was observed between lung controls and lungs treated with free antibiotic. In lungs treated with free antibiotic, 5.01 and 2.07 mg of tobramycin per pair of lungs were found respectively 1 and 3 h. after treatment as determined by HPLC analysis (Fig 2). In lungs treated with liposome-tobramycin, only traces of tobramycin, inferior to the threshold of quantitative HPLC determination, were detected. At the end of the experiment, either 16 h. after treatment, a mean of 31.72 mg per pair of kidneys were detected in animals treated with free tobramycin comparatively to a mean of 15.74 mg in animal treated with liposome-tobramycin. Traces of antibiotic inferior to the threshold of quantitative sensitivity of HPLC assay were detected only in the sera of animals treated with free antibiotic until 6 h. after treatment (Fig 2).

Discussion.

The eradication of mucoid *P. aeruginosa* in an animal model of chronic pulmonary infection has been previously demonstrated following the intratracheal administration of fluid liposome-tobramycin in liquid form (9). In view of a possible clinical development, different ways of liposome administration have to be tested. The aerosol administration of liposomes as dry powder is attractive for reasons related to the stability of the product and simplicity of the administration using dry powder

inhalation system (DPI's) and/or metered dose inhalers (MDI). In a recent work, it was demonstrated that fluid liposome-tobramycin used directly as dry powder in culture media, showed greater bactericidal efficiency than free tobramycin (17 to 50 times more efficiency) against *P. aeruginosa, Burkholderia cepacia, Xanthomonas maltophilia* and *Escherichia coli* (8). In the present work, an intratracheal aerosolizer containing a 600 mg-dose of powdered liposome or powdered tobramycin was used to treat pulmonary chronical infection in rats. The results presented here show that 16 h post treatment, powdered liposome-tobramycin aerosolized in infected rats killed 300 times more bacteria than the same dose of free antibiotic. The quantity of tobramycin detected by HPLC analysis in lungs, excluding the trachea, was 5.01 and 2.07 mg/pair of lungs, one and three hours respectively following the administration of free antibiotic. After the administration of liposome-tobramycin in powder form, only traces of tobramycin were detected in lungs but they were maintained until 16 h after treatment. This suggests that aerosolized powder form of liposome-tobramycin, because of hygroscopic problems, was less efficient than free antibiotic to reach the pulmonary tissue but showed longer persistence and a bactericidal efficiency 300 times more important in term of number of cfu than free antibiotic. This study also showed that less tobramycin is concentrated in kidneys following the administration of liposome-encapsulated tobramycin.

At first view, it may appear that the liquid form of fluid liposome-encapsulated tobramycin was more effective than the powder form since the same apparent quantity of encapsulated tobramycin in liquid form succeed to eradicate mucoid *P. aeruginosa* (8, 9). Nevertheless, to compare adequately the bactericidal activity of the liquid and the powder forms of liposomes every parameter influencing the

accurate dose of the encapsulated tobramycin really available to the lungs had to be examined. The usual preparation of liquid liposomes comprises a rehydration step followed by washing of liposomes to eliminate unencapsulated antibiotic and HPLC quantification of the encapsulated tobramycin is performed following this practice. On the other hand, a precise quantity of tobramycin is added to phospholipids and because liposomes are used as powder, unencapsulated tobramycin cannot be eliminated and must be considered in the total therapeutic dose. However, studies performed to determine the quantity of antibiotic effectively encapsulated in liposomes following direct incorporation of powdered liposome-tobramycin in medium culture at 37°C, have shown a mean of 73.5% of encapsulation. In this study, it was also determined that 57.3% of the powdered liposome-tobramycine dose leaves the microspayer comparatively to 97.1% for free antibiotic. The lower efficacy of the microspayer to deliver high percentage of the powdered liposomes comparatively to powder antibiotic was probably due to hygroscopic characteristic of the liposomal preparation which could be corrected if interest for this form of administration is maintained. These observations suggest that a mean of 236.8 mg of encapsulated tobramycin was administered to rats comparatively to 582.6 mg of free antibiotic. Finally, another important point to be considered between the previous study using liquid form of liposomes and the present study using powder form of liposomes, are the number and the duration of the treatments. In the present study, only one treatment was administered on a period of 16 hours comparatively to two and three treatments on a period of 32 and 48 hours in the previous study. If we consider all those parameters, the results obtained in this study suggest that aerosolized fluid liposome-tobramycin used as dry powder may be probably as effective as the liquid form to eradicate chronic pulmonary infections caused by *Pseudomonas* and related bacteria if aerosol

administration is optimized. Considering all these restrictions that could be corrected, this work suggests that the administration of fluid liposome-antibiotic as powder preparation may be of great efficiency in the treatment of CF patients colonized by *Pseudomonas* and other related bacteria. Moreover, if we consider the inefficiency of free antibiotic, these results suggest that fluid liposomes can fight the outbreak of different forms of antibiotic resistance particularly the low outer-membrane permeability which can arise as a stable or as an adaptive unstable origin as described in introduction.

Acknowledgement. This study was supported by a grant from the Canadian Cystic Fibrosis Foundation.

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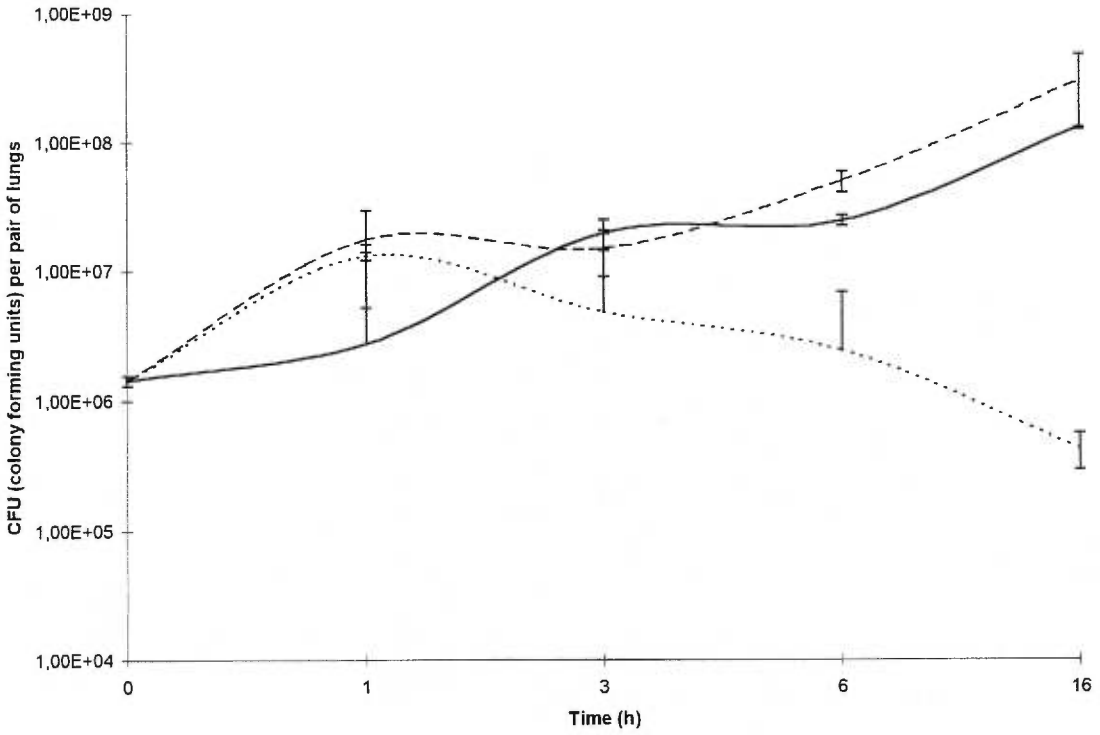

Figure 1. Bactericidal effect of fluid liposome-Tobramycin (600 mg) administered as dry powder aerosols to the rat model chronically infected with *Pseudomonas aeruginosa*. CFU counts in lungs were enumerated 0, 1, 3, 6 and 16 h post treatment. (—) bacteria exposed to phosphate buffered saline, (-----) bacteria exposed to free tobramycin; (- - -) bacteria exposed to dry powder liposome-tobramycin. Results are expressed as the arithmetic mean of the three samples ± S.E.M.

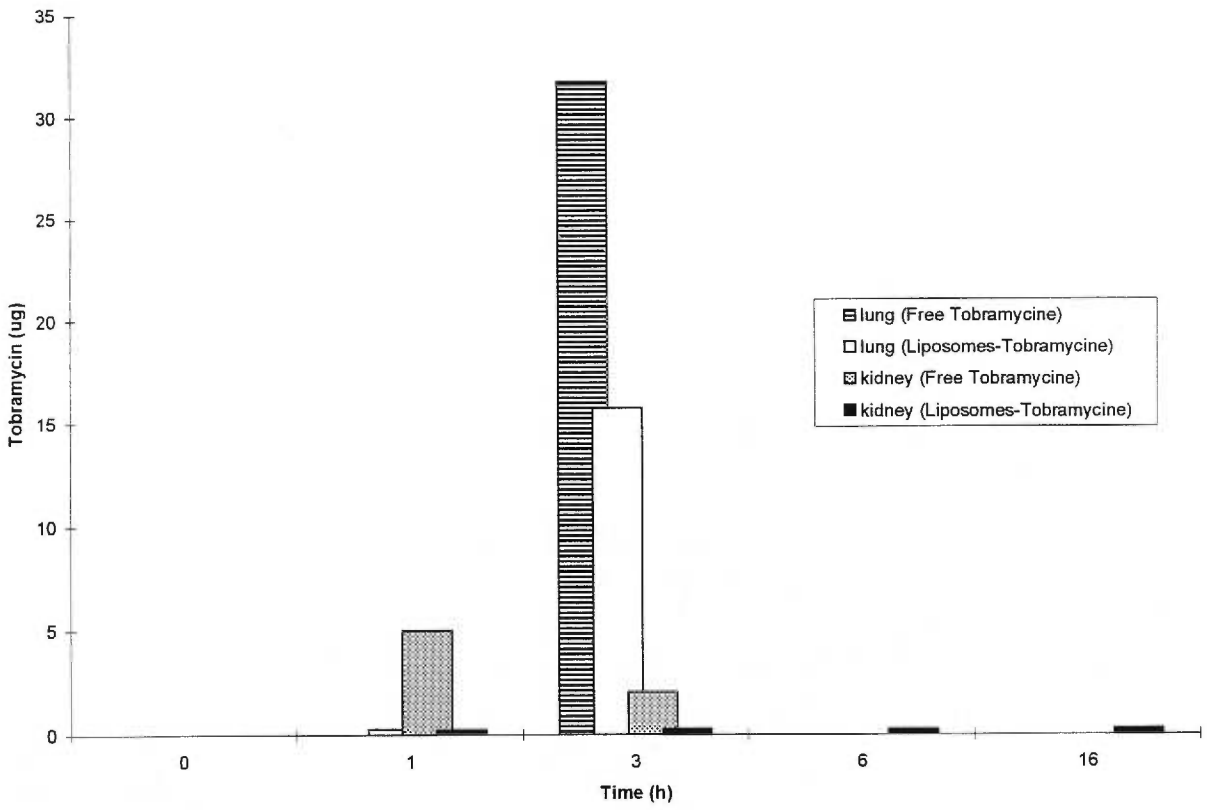

Figure 2. HPLC quantification of tobramycin in lung and kidney homogenates following aerosol administration of 600 mg of free and liposome-tobramycin as dry powder preparations. Analysis were performed 0, 1, 3, 6 and 16 h post treatment.

DISCUSSION ET CONCLUSION

VI DISCUSSION ET CONCLUSION

Les liposomes, ces vésicules constituées de phospholipides qui permettent d'encapsuler diverses molécules, ont été utilisés au cours des dernières années selon différentes modalités. Les liposomes thérapeutiques dits "classiques", sont de type rigide c'est-à-dire qu'ils sont constitués par un ensemble de phospholipides caractérisés par une T_C relativement élevée. Ces liposomes sont reconnus pour diminuer la toxicité des drogues qu'ils transportent mais à quantité égale, ils ne sont pas plus efficaces que l'antibiotique libre (Yardley *et al.*, 1997; Omri *et al.*, 1994). Ils sont utilisés expérimentalement et/ou commercialement dans le traitement d'infections à *Leishmania* (Alvin *et al.*, 1980), d'infection fongiques (Lopez *et al.*, 1983), d'infections à mycobactéries (De Marie *et al.*, 1994) et de cancer (Gabison *et al.*, 1989). Ces liposomes de type rigide nécessitent l'implication des cellules du système réticulo-endothélial pour rendre le médicament disponible aux micro-organismes.

Les premiers travaux "*in vivo*" réalisés dans notre laboratoire étaient basés sur une formulation de type rigide qui a augmenté la persistance de l'antibiotique au niveau pulmonaire tout en diminuant la toxicité de la molécule encapsulée. (Omri *et al.*, 1994). Bien que l'antibiotique encapsulé ait persisté pendant un minimum de 16 hrs, comparativement à moins d'une heure pour l'antibiotique libre, son efficacité bactéricide ne fut pas augmentée même si la quantité d'antibiotique détectée dans les poumons de l'animal était supérieure à la CMI de la bactérie (Omri *et al.*, 1984). Les études ultérieures ont été orientées vers le développement de nouvelles formulations dont les T_C s'échelonnaient de 29 °C à 46 °C (Beaulac *et al.*, 1997). Des études *in vitro* et *in vivo* de cinétique de libération et de persistance de l'antibiotique, ont permis de sélectionner deux formulations liposomales

prometteuses (Beaulac *et al.*, 1997). Une de ces formulations soit celle constituée de DPPC et de DMPG a réussi à éradiquer les infections pulmonaires chroniques causées par un *P. aeruginosa* muqueux (Beaulac *et al.*, 1996). Des études réalisées par un membre de notre équipe suggèrent fortement que l'efficacité thérapeutique particulière de ces liposomes fluides repose sur leur capacité d'amplifier la pénétration des antibiotiques dans la cellule bactérienne suite à une interaction directe des liposomes avec la paroi bactérienne (Sachetelli *et al*, 1998 en préparation). Cette caractéristique originale semble principalement due à la T_C de cette formulation liposomale qui se situe entre 29.5 et 33 °C en fonction du ratio DPPC/DMPG utilisé. Une T_C basse permettrait à la fois une interaction directe avec les cellules bactériennes et une libération modulée du médicament à 37 °C sur une période d'environ 16 hrs (Beaulac *et al.*, 1996; Beaulac *et al.*, 1998a ; Beaulac *et al.*, 1998b). L'originalité et le potentiel thérapeutique des liposomes fluides développés par notre laboratoire sont maintenant reconnus suite à l'obtention d'un brevet américain en janvier 1997 et la permission de passer en phase pays pour l'obtention d'un brevet international (PCT) en juin 1997.

Les travaux ultérieurs présentés dans ce travail, ont permis de mettre en évidence que les liposomes fluides sont également efficaces contre différentes souches bactériennes qu'elles soient ou non résistantes aux antibiotiques. Ainsi, la souche *P. aeruginosa* (PA # 429) qui possède une forte résistance vis-à-vis la tobramycine (CMI = 60 µg/ml), deux souches apparentées à cette dernière, soit *B. cepacia* et *S. maltophilia*, et deux souches bactériennes non apparentées, *E. coli* et *S. aureus* se sont avérées très sensibles à la tobramycine encapsulée et ceci à des concentrations inférieures de moitié à la concentration minimale inhibitrice (CMI) (Beaulac *et al.*, 1998a). En fait, la tobramycine encapsulée a

démontré un pouvoir bactéricide de 60 à 10^5 fois plus efficace que la même quantité d'antibiotique libre dépendamment de la bactérie testée (Beaulac *et al.*, 1998a). Un tel accroissement de l'activité bactéricide de l'antibiotique encapsulé ne peut s'expliquer que par une amplification de la pénétration de l'antibiotique dans la cellule bactérienne.

Compte tenu de l'intérêt de plus en plus marqué pour l'administration par aérosols de médicaments sous forme de poudre sèche destinés au système respiratoire, la possibilité d'administrer les liposomes sous leur forme lyophilisée a été analysée. Dans ce but les expériences réalisées précédemment avec la forme liquide des liposomes fluides ont été répétées directement avec la forme lyophilisée des liposomes associés à la tobramycine. Ces études ont montré que comparativement à la même quantité de tobramycine libre utilisée sous forme de poudre, la forme lyophilisée associée aux liposomes fluides provoquait une amplification importante du pouvoir bactéricide. Comparativement à l'antibiotique libre, l'activité bactéricide des liposomes associés à la tobramycine était supérieure de 47 fois pour *B. cepacia*, 17 fois pour *P. aeruginosa*, 40 fois pour *S. maltophilia* et 50 fois pour *E. coli* alors qu'il n'y avait pas d'amplification significative vis-à-vis *S. aureus*. (Beaulac *et al.*, 1998b). Si au premier abord, ces résultats semblent moins importants que ceux obtenus à l'aide des liposomes sous forme liquide, il faut tenir compte du fait qu'avec les liposomes en poudre, l'antibiotique non associé aux liposomes n'est pas éliminé comme c'est le cas lors de la préparation des supensions liposomales liquides. Lors d'études visant à mesurer le taux d'incorporation de la tobramycine aux liposomes en poudre dans les mêmes conditions que celles décrites pour la réalisation des études d'efficacité (Beaulac *et al.* 1998c), on a déterminé qu'avec des concentrations de tobramycine d'environ 600 µg, 73% de l'antibiotique

s'associait aux liposomes (Beaulac *et al.* 1998c) alors qu'à des concentrations de l'ordre de celles utilisées pour les études *in vitro* (1-30 µg/ml), le pourcentage d'association était d'environ 10% (résultats non montrés). Si l'on considère que près de 90% de l'antibiotique est encapsulé lorsqu'il s'agit des préparations liquides de liposomes, les résultats obtenus dans ces conditions expérimentales sont très positifs. De plus, il va s'en dire que les doses thérapeutiques qui seront ultérieurement utilisées chez l'humain se situeront obligatoirement dans la marge du 73% d'encapsulation.

L'effet bactéricide de la tobramycine associée aux liposomes aérosolisés sous forme de poudre, lorsque testé sur un modèle animal d'infection chronique à *P. aeruginosa,* a entraîné une baisse du nombre de bactéries intra-pulmonaires d'environ 300 fois comparativement aux animaux ayant reçu la même dose d'antibiotique libre. Cependant, il n'y a pas eu éradication de l'infection tel qu'observé antérieurement avec la préparation liposomale liquide (Beaulac *et al.*, 1996). Ces résultats doivent cependant être interprétés à la lumière de différences méthodologiques: lors de la présente étude, un seul traitement de 600 µg d'antibiotique a été administré comparativement à deux et trois traitements dans le cas de l'étude impliquant la préparation liposomale liquide. De plus, l'expérience réalisée à l'aide des liposomes lyophilisés s'est étendue sur une période de 16 hrs comparativement à 32 et 48 hrs dans le cas des expériences réalisées à l'aide des préparations liposomales liquides. D'autre part, il faut prendre en considération que la préparation liquide de liposomes avait été traitée pour éliminer l'antibiotique non encapsulé ce qui n'est pas possible avec la préparation lyophilisée. Ainsi, selon nos analyses, 73% ou 438 µg de tobramycine aurait été associés aux liposomes en poudre comparativement à 600 µg dans le cas des liposomes liquides. Finalement, certains problèmes

d'hygroscopie liés aux liposomes utilisés sous forme de poudre auraient limité à 57% (selon une étude statistique) la quantité d'antibiotique associée aux liposomes qui ont été projetés hors du microspray comparativement à 97% dans le cas de l'antibiotique libre. Prenant en considération ces différents paramètres et le fait qu'il est possible de développer des solutions au problème d'hygroscopie, les résultats obtenus suggèrent que la forme lyophilisée des liposomes associés à la tobramycine pourrait probablement être aussi efficace que la forme liquide pour l'éradication des infections pulmonaires chroniques causées par les *Pseudomonas.*

En raison de l'intérêt thérapeutique des liposomes fluides pour le traitement des patients atteints d'infections pulmonaires chroniques, il était important de tester différentes méthodes d'administration des liposomes par aérosols. Dans un premier temps, le maintien de l'intégrité des vésicules liposomales fluides suite à leur passage à travers un appareil de nébulisation a été analysé. Cinq nébulisateurs différents ont été sélectionnés pour réaliser ces études préliminaires de nébulisation, le Ventstream, le Sidestream, le Pari LC jet et le nébulisateur à ultrasons Ultrasonic 5000. Le Pari LC jet Star a été utilisé lors d'une étude ultérieure. Ces néblisateurs ont été retenus en raison de leurs performances (Clay *et al.*, 1983; Newman *et al.*, 1986; Newman *et al.*, 1987; O'Doherty *et al.*, 1988; Waldrep *et al.*, 1994; Niven *et al.*, 1992; Niven *et al.*, 1991; Niven *et al.*, 1990; Gilbert *et al.*, 1988; Gilbert *et al.*, 1992; Waldrep *et al.*, 1997). Alors que les deux premiers nébulisateurs n'ont pas réussi à maintenir l'intégrité de plus de 30% de liposomes nébulisés, il a été clairement démontré que de 55 à 68 % et 51 à 63 % de l'antibiotique demeurait encapsulé lorsque l'on utilisait respectivement le Pari LC Jet et le nébulisateur à ultrasons. Le Pari LC Jet a été finalement sélectionné car les études

complémentaires du Dr Coates ont montré l'obtention d' un *output* satisfaisant avec cet appareil dans des conditions semblables à celles observées chez les patients atteints de FK. Par sa conception, le nébulisateur ventillé Pari Lc Jet permet à l'air de circuler à travers la chambre du nébulisateur en augmentant le"*output*" alors que les nébulisateurs à jets non ventillés, livrent l'aérosol directement dans une pièce en forme de T. Dans ces conditions, contrairement au Pari LC jet, la performance respiratoire du patient n'a pas d'influence sur le "*output*" de l'appareil (Coates *et al.*, 1997). Le nébulisateur ultrasonique n'a pas été retenu en raison de son utilisation moins satisfaisante lors d'études préliminaires effectuées par l'équipe du Dr Coates.

Les travaux de caractérisation particulaire des aérosols à partir des liposomes fluides nébulisés à l'aide du Pari LC Jet ont été réalisés en collaboration avec le Dr Coates et les analyses par HPLC des échantillons préparés par notre laboratoire ont été effectuées par les Drs Dale Meisner et Elizabeth Kwong de MERCK FROSST. La distribution et le diamètre des particules d'aérosols ont été mesurés à l'aide d'un "LASER PARTICLE SIZER" et d'un impacteur à cascade. Toutes les précautions ont été prises afin de minimiser au maximum les erreurs qui peuvent se glisser dans ce genre de mesures, principalement en s'assurant que l'impacteur ne s'assèche jamais tout en contrôlant les paramètre d'inertie reliés à l'appareillage. Le Pari LC Jet était relié à un compresseur Pulmo-Aide réglé à des vitesses d'inspiration échelonnées entre 0 et 20-30 litres / min. afin d'évaluer la masse médiane moyenne du diamètre (MMAD) des gouttelettes d'aérosols générées dans ces conditions. Des échantillons d'aérosols étaient également collectés pour évaluer la stabilité des liposomes dans ces mêmes conditions. Ces études effectuées à partir de liposomes extrudés (400 nm) et non extrudés ont également permis de démontrer que la filtration des liposomes par extrusion n'était pas nécessaire

puisque les MMAD des gouttelettes d'aérosols ainsi que la stabilité des liposomes extrudés et non-extrudés n'étaient pas significativement différentes. Les études ont montré que les MMAD des gouttelettes d'aérosols formées à partir de la tobramycine libre et de la tobramycine encapsulée dans les liposomes fluides avant et après extrusion (diamètre de 0,4 μm) étaient respectivement de 4.11 +/- 0.22, 4.98 +/- 0.13 et 4.65 +/- 0.19 pour des vitesses d'inspiration de 20 L/min. en utilisanet un nébulisateur de type PARI LC JET (Macneish *et al.*, 1998, en préparation). Lorsque les préparations liposomales sont nébulisées à l'aide du PARI LC JET STAR, un nouveau prototype sur le point d'être mis sur le marché, les MMAD des gouttelettes d'aérosols formées à partir des liposomes fluides avant et après extrusion (diamètre de 0,4 μm) étaient respectivement de 3.85 +/- 0.12, et 3.98 +/- 0.03 pour des vitesses d'inspiration de 30 L/min. La valeur de 20-30 L/min. est mentionnée ici parce que les patients FK présentent des vitesses d'inspiration moyenne de 23 +/- 8.83 L / min. Ces résultats indiquent donc que les gouttelettes d'aérosols formées à partir des liposomes ont une MMAD compatible avec une distribution en profondeur dans le poumon puisque leur diamètre est inférieur à 5 μm. Cette étude a également montré le maintien de l'intégrité des vésicules fluides avec le nouveau nébuliseur puisque le pourcentage d'antibiotique encapsulé à la fin de la nébulisation se situait entre 45 et 70 %. Ces résultats sont présentés en annexe 1 et feront l'objet d'une publication conjointe avec l'équipe du Dr Coates.

La démonstration que la tobramycine encapsulée dans des liposomes fluides permette d'éradiquer des infections chroniques alors qu'à l'état libre cette dernière n'est pas plus bactéricide que de la saline,

démontre son importance dans la lutte contre la résistance microbienne aux antibiotiques. Cette propriété est également confirmée par la capacité de l'antibiotique encapsulé de démontrer une activité bactéricide importante à des concentrations inférieures au MIC. Ces résultats obtenus avec les liposomes fluides suggèrent fortement que ces derniers pourraient être utilisés pour lutter contre le phénomène de résistance bactérienne aux antibiotiques. Ces résultats sont d'autant plus probants que *P. aeruginosa* constitue un modèle bactérien exemplaire de résistance aux antibiotiques en raison de la faible perméabilité non spécifique de sa membrane externe (Angus *et al.*, 1982; Yoshimura *et al.*, 1982) et de la présence chez cette bactérie de pompes à reflux permettant à l'expulsion des drogues (Pooles *et al.*, 1993; Lewis, 1994; Nikaido, 1994). On peut également retrouver chez cette bactérie les autres phénomènes de résistance connus tels l'acquisition ou la dérépression du gènes de la β-lactamase ou autres enzymes inactivateurs d'antibiotiques tel le DNA-gyrase (Livermore *et al.*, 1987; Livermore *et al.*, 1989; Jacoby *et al.*, 1979), et l'altération de protéines cibles pour les antibiotiques telles les "*penicillin-binding proteins*" (PBP) et la dihydrofolate reductase. Tous ces mécanismes peuvent agir en synergie, l'augmentation de l'imperméabilité membranaire et l'action des pompes à reflux jouant un rôle prépondérant chez *P. aeruginosa* (Chen *et al.*, 1995). Toutefois, une autre cause de résistance bactérienne aux antibiotiques, qui serait largement sous-estimée, semble être un défaut de perméabilité adaptatif et instable qui a été bien documenté chez *P. aeruginosa* suivant une exposition aux aminoglycosides (Gilleland *et al.*, 1989; Barclay *et al.*, 1992). Cette résistance est dite réversible puisqu'elle nécessite la présence continue de l'antibiotique pour s'exprimer et se maintenir. Cette résistance, suite à une première exposition aux antibiotiques, serait en relation avec une régulation négative réversible du passage des drogues dans la bactérie, spécialement durant la période

de transport énergie dépendante (Daikos et al., 1990). Ce phénomène a été bien documenté par des études à la fois *in vitro* et *in vivo* chez le modèle murin d'infection à *P. aeruginosa* suivant l'exposition à un aminoglycoside (Gilleland, 1988; Daikos et al., 1990; 1991; Barclay et al., 1992). Récemment, la résistance adaptative à l'action bactéricide de la tobramycine a été identifiée chez *P. aeruginosa* dans les expectorations de 7/7 patients FK analysées rapidement suite à une antibiothérapie par inhalation (Barclay et al., 1996). Les facteurs suivants démontrent qu'une telle résistance n'est pas due à la sélection de souches mutantes: la réversibilité rapide de la résistance en absence de l'antibiotique, la nature constante du MIC des bactéries obtenues avant, durant et après la période de résistance et la stabilité des souches démontrée par des techniques sensibles de "fingerprinting" (Barclay et al., 1996). Parce que la résistance n'est maintenue qu'en présence continue de l'antibiotique, l'importance du rôle joué par ce type de résistance est difficile à évaluer et demeure largement inconnu. Il est possible que ce mécanisme adaptatif de résistance aux antibiotiques soit une cause significative d'échec de nombreux traitements contre des souches bactériennes qui une fois isolées s'avèrent sensibles à ces mêmes antibiotiques. A la lumière de ces faits et de la démonstration de l'efficacité bactéricide de la tobramycine encapsulée dans les liposomes fluides chez le modèle animal d'infection chronique à *P. aeruginosa* (Beaulac *et al.*, 1996), il semble que cette forme d'antibiothérapie ait la capacité de contrer le phénomène de résistance "instable et adaptative" aux antibiotiques.

La technologie des liposomes fluides en facilitant la pénétration des antibiotiques dans les cellules bactériennes, pourrait être en mesure de contrecarrer non seulement les phénomènes membranaires de résistance aux antibiotiques , mais également de diminuer les effets des autres formes de résistances

en raison du phénomène de synergie. Des travaux sont présentement en cours dans notre laboratoire afin de vérifier cette possibilité. Dans un avenir rapproché, des études de toxicité seront réalisées dans des laboratoires indépendants dans le but de vérifier l'innocuité des liposomes fluides. Suite à ces études pré-cliniques et cliniques 1, la tobramycine encapsulée dans les liposomes fluides, pourra être testée en tant que "drogue orpheline", pourra être testée chez des patients atteints de fibrose kystique colonisés par *B. cepacia*. Ultérieurement, les liposomes fluides devraient constituer une réponse appropriée aux différentes formes d'infections chroniques affectant le système respiratoire. Il s'agit d'une approche simple, efficace et économique qui permettra de redonner une seconde vie à de nombreux médicaments.

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ANNEXE 1

Tableau 1: Stabilité des liposomes exprimée en tant que pourcentage de la tobramycine toujours encapsulée suite à la nébulisation dans les appareils suivants:

"Pari LC JET"	55 à 68 % *
"Vent Stream"	31 à 37 % *
"Side Stream"	22 à 67 % *
"Ultra Sonic 5000"	51 à 63 % *

*: Pour des concentrations de tobramycine variant entre 157.84 et 3946 μg/ml.

Tableau 2: Détermination du diamètre moyen de la masse médiane (MMAD) des particules d'aérosols nébulisées à différentes vitesses d'inspiration (VI*).

Nébuliseur PARI LC JET

	VI = 0	VI = 10	VI = 20
Liposomes extrudés	5.72 +/- 0.26	5.17 +/- 0.24	4.79 +/- 0.19
Liposomes non extrudés	6.20 +/- 0.40	5.74 +/- 0.20	4.98 +/- 0.13

Nébuliseur PARI LC JET STAR

	VI = 0	VI = 15	VI = 30
Liposomes extrudés	4.09 +/- 0.18	3.91 +/- 0.09	3.98 +/- 0.03
Liposomes non extrudés	3.89 +/- 0.18	3.86 +/- 0.07	3.85 +/- 0.12

*: La vitesse d'inspiration était déterminée par un montage où la pression et le taux d'humidité étaient contrôlées.

Tableau 3: Stabilité des liposomes exprimée en tant que pourcentage de la tobramycine toujours encapsulée suite à la nébulisation d'une solution liposomale contenant 1 mg/ml d'antibiotique.

	PARI LC JET	PARI LC JET STAR
Liposomes extrudés	56 %	66 %
Liposomes non extrudés	68 %	50 %

ANNEXE 2

J. MICROENCAPSULATION, 1997, VOL. 14, NO. 3, 335–348

In vitro kinetics of drug release and pulmonary retention of microencapsulated antibiotic in liposomal formulations in relation to the lipid composition

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(*Received 15 December 1995; accepted 15 February 1996*)

In previous *in-vivo* studies, we demonstrated that liposomal entrapment of tobramycin resulted in an increased availability of the antibiotic in the lungs without increasing bactericidal efficacy (Omri *et al.* 1994). With the aim of developing liposomal formulations allowing more efficient liposome-bacteria interactions, we studied the influence of lipid composition on both drug release and pulmonary retention of encapsulated tobramycin. The phase transition temperatures of nine liposome-tobramycin formulations consisting of two synthetic phospholipids (distearoyl phosphatidylcholine (DPSC) or dipalmitoyl phosphatidylcholine (DPPC) with dimyristoyl phosphatidyl-glycerol (DMPG) or dimyristoyl phosphatidylcholine (DMPC)) were determined by differential scanning calorimetry. Liposomes, varying in terms of membrane fluidity and charge were submitted to *in-vitro* and *in-vivo* kinetic studies while retention and release of tobramycin were measured by high-performance liquid chromatography (HPLC). Five less fluid liposome formulations showed absence or very low tobramycin release in *in-vitro* tests and long term pulmonary retention of tobramycin. Four fluid liposome formulations showed in *in vitro* tests modulated tobramycin release while pulmonary retention of tobramycin was dependent of the presence of charged phospholipids. Administration of charged fluid liposomes in mice showed a low level of tobramycin in the kidneys; non-charged fluid liposomes exhibited a relatively high level of tobramycin retention in the kidneys.

Keywords: Pulmonary, drug release, liposome, antibiotic, microencapsulated.

Introduction

Encapsulation of bioactive compounds in natural or synthetic matrices has been extensively studied over the past decades (Gregoriadis and Florence, 1993). Such a strategy of administration may allow controlled retention of encapsulated drug, protection from the inactivation or degradation of the bioactive compound, reduction of the risk of toxicity and selective delivery of antibiotics.

Numerous *in-vivo* studies, particularly in the cancer and antimicrobial therapies, have demonstrated that some liposome-entrapped drugs exhibit superior pharmacological properties to those observed with conventional formulations (Lopez-

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Berestein and Fidler 1989). Liposome-entrapped drugs against microbial diseases are used primarily because of the toxicity and/or inability of potent agents to reach infected intracellular sites effectively. There are numerous evidences that liposome formulations are superior to free antimicrobial agents both in terms of distribution to the relevant intracellular sites and therapeutic efficacy (Barsoum and Reich 1982, Dees *et al.* 1985, Lopez-Berestein *et al.* 1987,Duzgunes *et al.* 1988, Swenson *et al.* 1990, Janoff 1992). Conversely, a few *in vivo* investigations with liposomes were reported against extracellular infections (Price *et al.* 1990, 1994, Assil *et al.* 1991, Di Rocco *et al.* 1992, Frucht-Perry *et al.* 1992, Omri *et al.* 1994). In the majority of such studies, *Pseudomonas aeruginosa* was the main targeted bacteria, probably because of the difficulties to treat *P. aeruginosa* infections in cystic fibrosis (CF), and in patients with immunosuppression, burns and malignancies.

In a previous work performed in an animal model of chronic pulmonary infection with *P. aeruginosa*, we demonstrated that liposome-entrapped tobramycin increased markedly the residence time of antibiotic in the lungs and reduced systemic drug absorption (Omri *et al.* 1994). However, the liposomal aminoglycoside formulation tested did not show improvement of bactericidal activity in the animal model when compared to free antibiotic. Such results suggested to us that the lipidic formulation used (disteroyl phosphatidylcholine and dimyristoyl phosphatidyl-glycerol at a 10:1 molar ratio), leads to the formation of vesicles too rigid to allow effective interaction between bacteria and encapsulated antibiotic.

By varying the lipid composition, charge, size and number of bilayers, the biophysical characteristics of liposomes can be controlled to influence drug release, drug persistence in the targeted organ and interaction with cells. Depending on the gel-liquid crystalline transition temperature of the phospholipids, liposomal membranes can attain various degrees of fluidity as a function of the temperature. Thus, using four different phospholipids, we designed liposomal formulations with different characteristics relative to the charge and membrane fluidity. The fluidity of the different formulations of liposome-encapsulated tobramycin was determined by differential scanning calorimetry (DSC). Afterward, selected formulations were submitted to *in vitro* and *in vivo* tests to determine how the lipid composition influences both the kinetics of drug release and the drug pulmonary retention.

Materials and methods

Animals

Balb-C mice (pathogen free, 6–8 weeks old, weighing approximately 25 g; Charles River, Saint-Constant, Québec, Canada) were used in experiments. Animals were housed three per cage and allowed free access to food and water. Mice were anesthetized with a mixture of 80 mg of ketamine hydrochloride per kg of body weight (Rogar/STB Inc., Montreal, Canada) and 1 mg of xylazine per kg (BAYVET Division, Chemagro Limited, Ontario, Canada) by intramuscular injection.

Table 1. Phospholipid characteristics.

Phospholipids	Molecular weight	Theoretical Tc† (°C)	Molecular charge
DSPC‡	79·15	55·0	none
DMPC*	677·85	23·0	none
DMPG§	688·85	23·0	negative
DPPC¶	734·05	42·0	none

* DMPG: Dimyristoyl phosphatidyl-glycerol
† Phase transition temperature
‡ DSPC: Distearoyl phosphatidylcholine
§ DMPC: Dimyristoyl phosphatidylcholine
¶ DPPC: Dipalmitoyl phosphatidylcholine

Preparation of liposomes

Liposomes were prepared with the following synthetic phospholipids: distearoyl phosphatidylcholine (DSPC), dimyristoyl phosphatidyl-glycerol (DMPG) dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC) (Avanti Polar Lipids Inc. Birmingham, AL, USA) (Table 1). Liposomes with the combinations DSPC/DMPG, DSPC/DMPC, DSPC/DPPC, DPPC/DMPG, and DPPC/DMPC (10:1 and 15:1 molar ratio) were prepared by a dehydration-rehydration vesicle (DRV) method (Kirby and Gregoriadis 1984, Lagacé *et al.* 1991) with modifications. Briefly, appropriate amounts of lipid mixture were dissolved in chloroform in a round-bottom flask and dried to a lipid film by roto-evaporation (Buchi Rotavapor-KRvr 65/45) at 65°C under vacuum (Buchi 168-Vacuum/Distillation controller). The lipids were then redissolved in phosphate buffered saline (PBS) 1:20 and lyophilized (Virtis Genesis 12EL Console freeze dryer, Canberra Packard, Ontario, Canada)) in ampoules at 4°C. Ampoules were kept at −70°C until use. After rehydration with tobramycin (Eli Lilly, Toronto, Ontario, Canada), liposomes were filtered in an extruder (Lipex Biomembranes Inc., Vancouver, B.C., Canada) charged successively with polycarbonate membranes of 1 µm, 0·6 µm and 0·4 µm. Control liposomes were prepared similarly but PBS was used instead of the antibiotic. Phospholipid dosage of each liposome preparation was performed by quantifying free inorganic phosphate from phospholipid mineralisation by a modified method of Fiske and Subbarow (Barlett 1958).

Analysis of Differential Scanning Calorimetry (DSC)

DSC was performed with a Hart differential scanning calorimeter (Hart Scientific, Pleasant Grove, Utah) equipped with three stainless steel ampoules. The first one contained 0·9 ml of free tobramycin, the second liposome alone, and the third liposome entrapped tobramycin. The scanning rate was 40°C/h for all experiments. Each scan spanned between 22·5 and 92·5°C. The base line was determined by using the buffer PBS used to prepare the liposomes. The temperature of maximal excess heat capacity was defined as the phase transition temperature.

In vitro *kinetics of drug release*

Liposomes were prepared as described above using 345 mg of phospholipids (0·436–0·469 mM) for the molar ratio of 15:1 and 220 mg of phospholipids (0·270–0·292 mM) for the molar ratio of 10:1. All liposomal formulations contained the same quantity of phospholipids as determined by the modified method of Fiske and Subbarow (Bartlett 1958). Liposomes were washed, suspended in 1·5 ml of PBS and incubated at 37°C with constant agitation (250 rpm). At various times (0, 1, 8, 24, 32 and 48 h), aliquots of 200 µl were collected and liposomes were pelleted by centrifugation (500 G X 20 min). Experiments were performed in duplicate. Supernatants (124 samples) and washed liposomes (124 samples) were conserved at −70°C in methanol extraction solutions before antibiotic quantification by HPLC. Drug release was calculated as a function of time.

Drug pulmonary retention

At least 126 mice were divided into 7 groups of 18 (3 mice per period of time) to test 5 liposomal formulations. Free tobramycin and PBS were administered as controls. Anaesthetized mice were given intratracheally either 50 µl of PBS, or 50 µl of free or liposome-encapsulated tobramycin (800 µg). Liposomes were prepared according to the following formulations: DSPC/DMPC 15:1, DPPC/DMPC 15:1 and 10:1, DPPC/DMPG 15:1 and 10:1. Intratracheal instillations (25-gauge, 10-mm catheter) were performed as described previously (Omri *et al.* 1994). At 0, 1, 8, 16, 32 and 48 h after instillation, groups of three mice were anaesthetized. The animals were then sacrificed and the entire lungs and both kidneys were removed aseptically, weighed, and then homogenized in cold sterile PBS (40% wt/vol) for 30 s with a Polytron Homogenizer (Polytron; Kinematica, Lucerne, Switzerland). The homogenizer was rinsed and flamed between samples. Homogenized tissues were conserved at −70°C in methanol extraction solutions before high-performance liquid chromatography (HPLC) analysis procedure.

HPLC analysis

The procedure for the determination of tobramycin in liposomes and in tissues was based on derivatisation of the tobramycin with 1-fluoro2,4-dinitrobenzene (FDNB) as described (Omri *et al.* 1994) with modifications. The tobramycin contained in homogenized tissue samples and in liposomes was extracted by methanol as follows: 100 µl of liposomes or homogenized tissue were added to 1 ml of methanol (MeOH), vortexed 1 min and incubated at 65°C for 30 min; 900 µl of PBS was then added, vortexed 1 min and centrifuged at 5000 × g for 20 min 4°C; 170 µl of supernatant was transferred in an ampoule containing 90 µl of FDNB prepared in MeOH (180 g/l), 60 µl of 0·1 M borate buffer, pH 9·3, and 670 µl of MeOH. Ampoules were sealed, vortexed and heated for 30 min at 80°C and then cooled at room temperature. As standards for liposome-encapsulated tobramycin analysis, tobramycin reference solutions were prepared in PBS with known amounts of tobramycin and processed as described above. As standards for the determination of tobramycin in tissues, known amounts of tobramycin were prepared in homogenized control tissues and treated as described above.

HPLC system with ultraviolet (UV) detection was used as previously described

(Barends *et al.* 1980, 1981a, b). For the quantitative analysis of tobramycin in liposomes collected during *in-vitro* kinetic analysis, the HPLC system comprised a pump, a system controller (model Varian 5000), a simple loop injector (Reodyne 7125), a UV spectrophotometric detector (Varian 2550) and a recorder (Spectra-Physics SP 4290). The separation was carried out on a Zorbax SB-C18 column (4·6 × 25 cm I.D.), particle size, 4 um (Du Pont Co.). For the quantitative analysis of tobramycin in tissues, the HPLC system comprised a pump (model 590, Millipore, Waters Chromatography Division, USA), a system controller (model TCM, Millipore), an auto-injector type WISP (model 710b, Millipore), an UV spectrophotometric detector (model 490, Millipore) and a Millenium 2010 chromatography system manager (Millipore). The separation was carried out on the same column as described above. The mobile phase was acetonitrile-MeOH-potassium phosphate buffer (10 mM) (65:10:25, v/v, pH 7·2) and the flow rate was 1·3 ml/min. The column effluent was monitored at 350 nm and at 0·005 absorbance units full-scale. Chromatography was performed at room temperature.

Calculations and statistics

All computer analyses were performed with a non-linear least-squares regression and a linear regression system using EXCEL and GRAF PAD PRISM data system. Results are expressed as ±SEM (standard error of means) of the indicated number of samples. *P*-values ⩽ 0·05 were considered significant.

Results

Differential scanning calorimetry

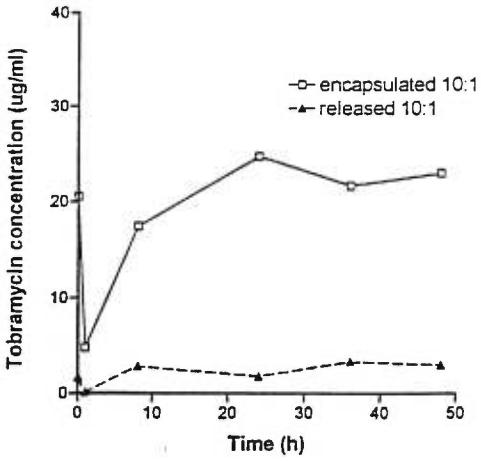
Preparations of free tobramycin, free phospholipid and liposome formulation with encapsulated tobramycin were analysed in parallel by DSC, yielding phase transition temperatures (table 2) from the thermograms. Each preparation showed a single sharp scan transition. Liposome preparations containing two types of phospholipids with encapsulated tobramycin showed a phase transition with two peaks: a main transition for the major constituent and a small pretransition for the

Table 2. Results of DSC† melting profiles.

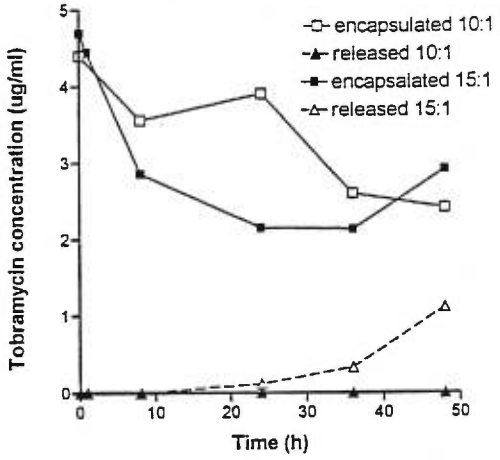
Formulation	Molar ratio	Tc (°C)‡
DSPC/DMPG	10·00:1	40·0
DSPC/DMPG	15·73:1	46·0
DSPC/DMPC	10·00:1	42·0
DSPC/DMPC	15·48:1	44·0
DSPC/DPPC	10·00:1	45·0
DSPC/DPPC	16·77:1	46·0
DPPC/DMPG	10·00:1	29·5
DPPC/DMPG	16·94:1	33·0
DPPC/DMPC	10·00:1	29·5
DPPC/DMPC	16·67:1	35·0

† Differential scanning calorimetry.
‡ Phase transition temperature.

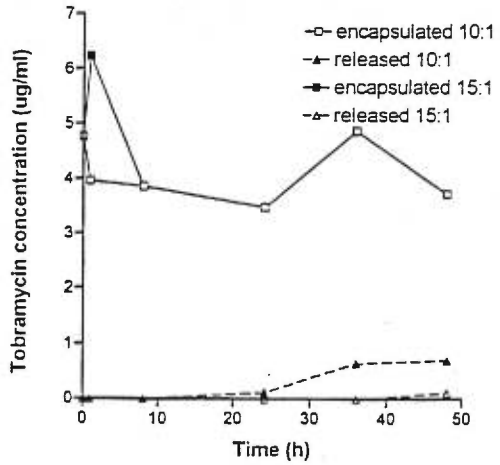
(A) DSPC / DMPG



(B) DSPC / DMPC



(C) DSPC / DPPC



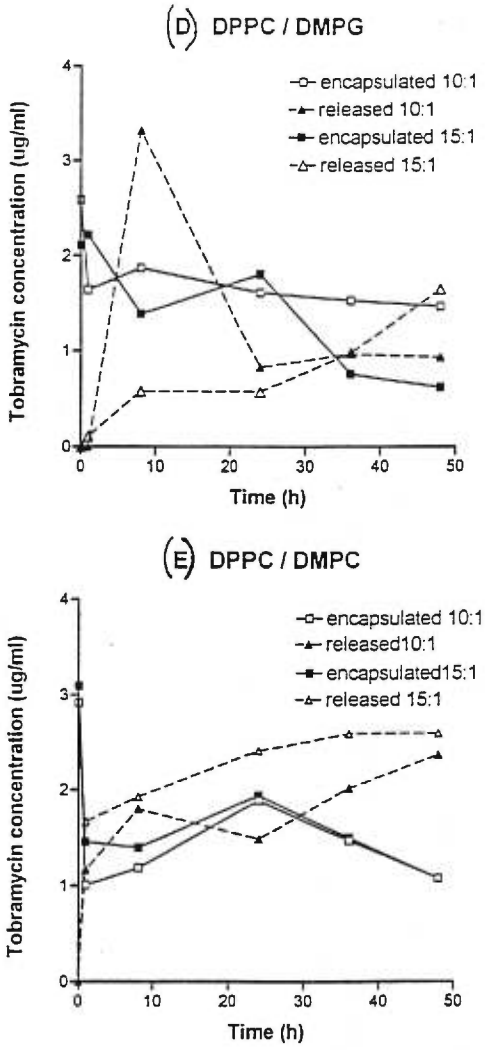
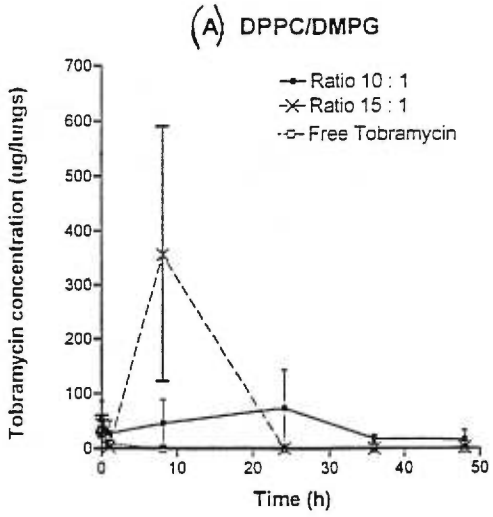


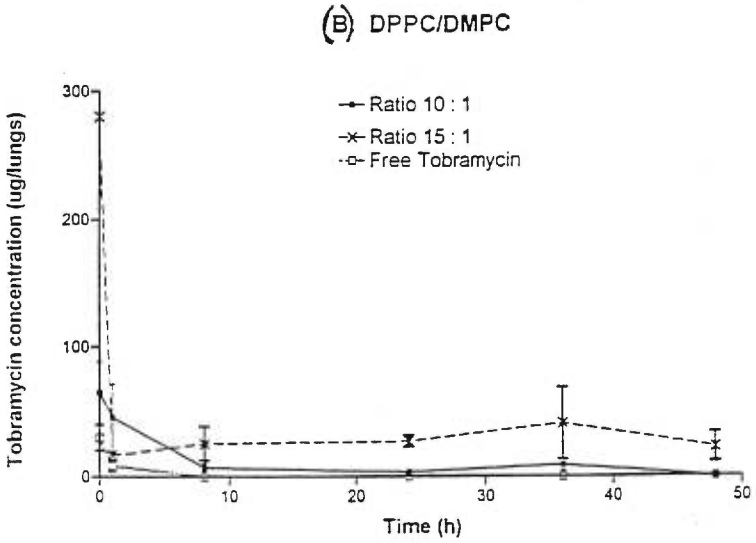
Figure 1. *In-vitro* kinetic study for determining the quantity of released tobramycin at 37°C for 9 liposomal formulations. Liposomes were prepared with the following combinations: (A) DSCP/DMPG (distearoyl phosphatidylcholine/dimyristoyl phosphatidyl-glycerol); (B) DSPC/DMPC (distearoylphosphatidylcholine/dimyristoylphosphatidylcholine; (C) DSPC/DPPC (distearoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine); (D) DPPC/DMPG (dipalmitoyl phosphatidylcholine/dimyristoyl phosphatidyl-glycerol); and (E) DPPC/DMPC (dipalmitoyl phosphatidylcholine/ dimyristoyl phosphatidylcholine). All liposomal formulations contained the same quantities of phospholipids. Tobramycin was quantified by high-performance liquid chromatography.

minor component. The peak of pretransition phase corresponded to the gel to liquid-crystalline transition leading to drastic change in the conformation of phospholipidic membranes. Alterations of DSC melting profiles for the phospholipid bilayers studied were only slightly modified in the presence of tobramycin.

In-vitro *kinetic of drug release and drug pulmonary retention*

Two major groups of liposomal formulations composed of non-charged phospholipids like DSPC or DPPC as the main phospholipids and of a non-charged or a negatively charged phospholipid as second phospholipid were analysed. The five formulations where DSPC was the main phospholipid showed high retention levels of encapsulated drug during the kinetic study, drug release being very low (figure 1a, b, c). More precisely, no free tobramycin was detected in supernatant following the assay with the formulations DSPC/DMPC at a molar ratio of 10:1 and DSPC/DPPC at a molar ratio of 15:1 (figure 1b, c). With the formulations DSPC/DMPC at a molar ratio of 15:1 and DSPC/DPPC at a molar ratio 10:1, the detection of free tobramycin was evident only 36 h after the onset of the study (figure 1b, c). In fact, only DSPC/DMPG formulation at a molar ratio of 10:1 showed noticeable liberation of free tobramycin but at a maximum of 14% compared to the encapsulated-liposome tobramycin (figure 1a). For this reason,





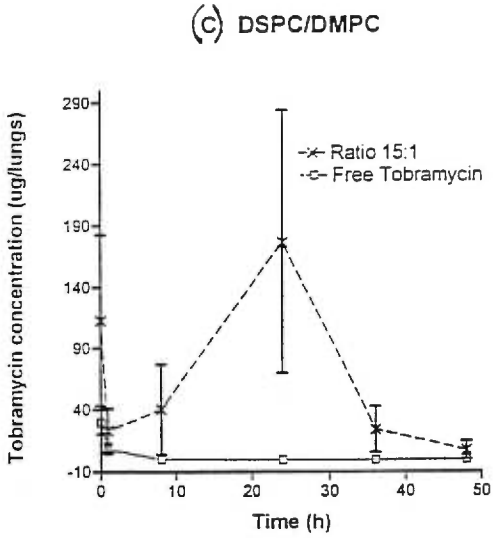


Figure 2. Determination of pulmonary retention in mice of liposome-encapsulated tobramycin by high-performance liquid chromatography after a single intratracheal administration of 5 liposomal formulations containing 800 µg of tobramycin. Liposomes were prepared with the following combinations: A: DPPC/DMPG (dipalmitoyl phosphatidylcholine/dimyristoyl phosphatidyl-glycerol); B: DPPC/DMPC (dipalmytoyl phosphatidylcholine/dimyristoyl phosphastidylcholine); C: DSPC/DMPC (disteroyl phosphatidylcholine/dimyristoyl phosphatidylcholine). Free tobramycin was used as control.

the *in vivo* persistence study was not performed with the DSPC group with the exception of DSPC/DMPC at a molar ratio of 15:1 used as a reference.

The four formulations where DPPC was the main phospholipid showed a very different pattern. We observed a consistent liberation of free tobramycin as a function of time beginning 1 h after the onset of the kinetic study with the DPPC/DMPC formulations at molar ratios of 15:1 and 10:1 (figure 1e). Otherwise, liberation of free tobramycin from the DPPC/DMPG formulations was delayed for 8h after the onset of the kinetic study and the liberation was more gradual for DPPC/DMPG at a molar ratio of 15:1 (figure 1d). When the DPPC formulations were tested for tobramycin pulmonary retention, the DPPC/DMPC formulation at a ratio of 10:1 showed a very low level of tobramycin retention (figure 2b) while the same formulation at a molar ratio of 15:1 showed a sustained tobramycin level of about 40 µg per pair of lungs (figure 2b). However, the DPPC/DMPC formulations showed a surprising level of tobramycin retention in kidneys reaching 61 µg per pair at 1 h for the DPPC/DMPC 15:1 formulation and 56 µg at 48 h with the DPPC/DMPC 10:1 formulation (table 3). The pulmonary retention of tobramycin with the DPPC/DMPG 15:1 formulation was significant 8 h after the intratracheal administration reaching a mean of 356 µg per pair of lungs (figure 2a). The individual values for each mouse were 799, 269 and 2 µg per pair of lungs. This experiment was repeated independently with three other mice and similar results were obtained: 479, 183 and 58 µg per pair of lungs. The pulmonary retention of tobramycin with the DPPC/DMPG 10:1 formulation was respectively 46 µg and 73 µg of tobramycin per pair of lungs 8 and 24 h after intratracheal

Table 3. Antibiotic quantification (μg/kidneys) in mice following administration of liposomes.

	Tobramycin concentration			
Formulations	Phospholipid Ratio 10:1		Phospholipid Ratio 15:1	
DSPC/DMPC TIME (h)	μg/kidneys	SEM†	μg/kidneys	SEM
1·00	not done		not done	
48·00	not done		12·72	3.83
DPPC/DMPC TIME (h)	μg/kidneys	SEM	μg/kidneys	SEM
1·00	9·26	4·63	61·48	24·98
48·00	56·61	7·46	27·65	16·81
DPPC/DMPG TIME (h)	μg/kidneys	SEM	μg/kidneys	SEM
1·00	19·27	3·82	30·05	5·44
48·00	8·86	4·64	10·80	0·98
Free tobramycin TIME (h)	μg/kidneys	SEM		
1·00	30·23	20·86		
48·00	7·52	4·66		

† SEM: Standard error of means.

administration of the drug (figure 2a). The level of tobramycin in the kidneys with this latter formulation was very low (<20 μg) when compared to the level of tobramycin detected in the lungs.

Discussion

In a recent model of experimental *P. aeruginosa* infection in rats, we previously demonstrated that intratracheal administration of liposome-encapsulated tobramycin compared with free tobramycin resulted in a prolonged pulmonary retention time of the antibiotic within the lungs of infected rats without enhancement in the therapeutic activity of the antibiotic (Omri *et al.* 1994). Taking into consideration the fact that the quanity of antibiotic in the lungs remained stable for the 16-h period studied, we hypothesized that the lipid composition of the vesicles was too rigid to allow effective interaction with bacteria. In the present study we examined the influence of the lipid composition of liposomes in terms of fluidity/rigidity and the presence of electrically charged phospholipid on the *in vivo* kinetics of drug release at 37°C and pulmonary retention of encapsulated tobramycin. We used nine types of multilamellar liposomes (mean diameter of 0·4 μm) composed of different combinations of four phospholipids DSPC, DMPG, DMPC, DPPC. Liposomal formulations were made of synthetic phospholipids because of their high purity and because they are less subject to oxidative degradation due to

the saturation of their fatty acyl chains (Konings 1984, Grit and Crommelin 1993).

Information about DSC melting profiles was obtained for the nine formulations after hydration with tobramycin (table 2). The phospholipidic phase-transition temperature of liposomes was minimally affected by tobramycin. The phase transition temperature of the nine formulations varied between 46 and 29·5°C thus allowing us to study the influence of different levels of membrane fluidity. DSC analysis showed that every formulation comprising DSPC as the main phospholipid presented a phase transition between 40 and 46°C. The membrane fluidity of such liposomal formulations when tested in *in-vitro* kinetic studies induced the release of minimal quantity of drug as compared to the quantity of encapsulated tobramycin during the 48-h period analysed (figure 1a, b, c). In fact, no tobramycin release was observed before 24 h from the DSPC/DMPC and DSPC/DPPC formulations and a maximum release of 14% was observed with the DSPC/DMPG formulation. After 36 and 48 h, a maximum of 14 and 27% of tobramycin were released respectively as compared to the encapsulated form of tobramycin. *In-vitro* kinetic studies performed with liposomal formulations where DPPC was the main phospholipid showed progressive and sustained tobramycin release during the whole 48 h period studied (figure 1d, e). Differences were noted between non-charged DPPC/DMPC and negatively charged DPPC/DMPG formulations: the two DPPC/DMPC formulations showed similar patterns of about 50% tobramycin retention-release until 36 h, but the release of tobramycin by the DPPC/DMPG formulations was more progressive. With the DPPC/DMPG formulations, detection of free tobramycin was first observed at 8 h compared to 1 h for the DPPC/DMPC formulations. Such a difference between the two groups of formulations exhibiting equivalent phase transition temperature may be explained by the presence of negatively charged phospholipid in the DPPC/DMPG formulation. Indeed, in pharmaceutical liposome formulations, a charged phospholipid tends to improve the physical stability of liposomes by reducing the rate of aggregation (Crommelin 1984, Grit and Crommelin 1993). Moreover, it was suggested that in the absence of a charge phospholipid, vesiculation is incomplete upon hydration of the cast lipid film (Talsma *et al.* 1992). A molar ratio of 10:1 for the DPPC/DMPG formulation induced significant release of tobramycin 8 h post administration while with a molar ratio of 15:1, drug release was slower and more progressive. These results may be consistent with the fact that the DPPC/DMPG 10:1 presents a phase transition temperature of 29·5°C compared to 33°C for the DPPC/DMPH 15:1 formulation.

Considering the low levels of released antibiotic and/or the high liposomal rigidity of DSPC formulations, pulmonary retention of tobramycin was evaluated only with one DSPC/DMPC formulation as a reference. Otherwise, the four DPPC formulations were submitted to *in-vivo* kinetic studies. The sustained pulmonary retention of tobramycin (>50 µg pair of lungs) observed until 24 h with the formulation DSPC:DMPG confirmed the higher stability of this formulation as previously described (Omri *et al.* 1994). The use of charged 10:1 DPPC/DMPG liposomal formulation induced a sustained level of tobramycin in the lungs for 24 h (mean of 55 µg pair of lungs). On the other hand, results obtained with the charged liposomal 15:1 DPPC/DMPG formulation were difficult to explain. Tobramycin was detected 8 h after liposome administration at very high levels in two out of three mouse lungs. This experiment was repeated a second time and

gave essentially the same results. Therefore, the persistence of high drug levels until 8 h after administration may be preferable to a longer time release if the fluidity characteristics of the formulation allows an increase in the interaction of the encapsulated drug with bacterial cells. Results obtained with the 10:1 DPPC/DMIPC formulation showed that this formulation did not slow *in-vivo* long term retention of tobramycin. A combination of absence of charge phospholipid and of a phase transition temperature of 29·5°C may explain the shortening of pulmonary retention of liposome-entrapped tobramycin.

The presence of tobramycin in mouse kidneys was also determined by HPLC 1 h and 48 h following liposome-entrapped tobramycin administration. The choice of these periods was determined by preliminary studies. The administration of encapsulated tobramycin in DPPC/DMPG formulations allowed detection of reduced quantities of tobramycin in kidneys compared to the quantities of tobramycin detected in the lungs. When tobramycin was encapsulated in DPPC/DMPC formulations, levels of tobramycin detected in kidneys were higher than with any other formulation. This suggests that the absence of charge phospholipids in liposomes may influence the increase of drug retention in the kidneys. The fact that the absence of charge phospholipid in liposomes tends to improve the rate of aggregation and formation of larger vesicles (Lichtenberg and Barenholz 1988, Tocanne and Teissie 1990, Talsma *et al.* 1992) may be a factor able to influence the drug retention in the kidneys.

These data suggest that when incorporated in less fluid liposomes, predominantly composed of DSPC, tobramycin is released very slowly compared to the fluid DPPC liposomes. Moreover, it is tempting to speculate that interactions between liposomes and bacterial cells may be impaired by high liposomal rigidity, as suggested by previous work (Omri *et al.* 1994). The use of fluid liposome predominantly composed of DPPC, with addition of a charged phospholipid, increase the release of free tobramycin while maintaining levels of tobramycin in the lungs. On the other hand, the absence of charged phospholipids in DPPC/DMPC formulations seems to increase fluidity in a way that does not allow long term pulmonary retention while inducing higher retention of tobramycin in kidneys. In conclusion, evidence is presented that lipid composition of liposomes seems to be an important determinant for the release, pulmonary retention, reduction of the risk of toxicity and hence the therapeutic effect of the encapsulated drug. Subsequent studies of the therapeutic value of encapsulated antibiotics in experimental infection models have shown the effective bactericidal effect of charged, fluid liposome tobramycin against chronic *P. aeruginosa* infections (Beaulac *et al.* 1996).

Acknowledgements

We gratefully acknowledge Dr Claude Gicquaud, Université du Québec à Trois-Rivières, for assistance with DSC methods. We thank Mrs Louise Paquette, Institut de Recherche en Biotechnologie, for her technical assistance in the HPLC analysis.

This study was partially supported by a grant from Fonds de la Recherche en Santé du Québec. Sébastien Clément-Major was the recipient of a studentship from the Canadian Cystic Fibrosis Foundation.

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ANNEXE 3

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1996, p. 665–669
0066-4804/96/$04.00+0

Eradication of Mucoid *Pseudomonas aeruginosa* with Fluid Liposome-Encapsulated Tobramycin in an Animal Model of Chronic Pulmonary Infection

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Received 13 July 1995/Returned for modification 4 September 1995/Accepted 21 December 1995

Despite controversies associated with forms and value of antibiotic therapy for cystic fibrosis patients, antibiotherapy remains a cornerstone in the management of those patients. Locally administered liposome-encapsulated antibiotics may offer advantages over free antibiotics, including sustained concentration of the antibiotic, minimal systemic absorption, reduced toxicity, and increased efficacy. We evaluated the efficacy of free and encapsulated tobramycin in fluid and rigid liposomal formulations administered to rats chronically infected with *Pseudomonas aeruginosa*. Chronic infection in lungs was established by intratracheal administration of 10^5 CFU of a mucoid variant of *P. aeruginosa* PA 508 prepared in agar beads. Antibiotic treatments were given intratracheally at time intervals of 16 h. After the last treatment, lung bacterial counts were determined and tobramycin levels in the lungs and kidneys were evaluated by high-performance liquid chromatographic analysis and microbiological assay. Two independent experiments showed that animals treated with encapsulated tobramycin in fluid liposomes had a number of CFU less than the minimal CFU number required to be statistically acceptable compared with $\geq 10^6$ CFU per pair of lungs for animals treated with encapsulated tobramycin in rigid liposomes, free antibiotic, or liposomes without tobramycin. Tobramycin measured in the lungs at 16 h after the last treatment following the administration of encapsulated antibiotic was still active, and its concentration was ≥27 μg/mg of tissue. Low levels of tobramycin were detected in the kidneys (0.59 to 0.87 μg/mg of tissue) after the administration of encapsulated antibiotic, while 5.31 μg/mg of tissue was detected in the kidneys following the administration of free antibiotic. These results suggest that the local administration of fluid liposomes with encapsulated tobramycin could greatly improve the management of chronic pulmonary infection in cystic fibrosis patients.

Most patients with cystic fibrosis (CF) by their second decade of life become colonized with *Pseudomonas aeruginosa* in their bronchial secretions (1, 14, 18, 22). Colonization with mucoid variants is considered the most important factor determining the prognosis for CF patients (13, 24, 30, 32). Antibiotherapy of CF is controversial because it acts more as a palliative than as a cure. However, it is recognized that antibiotic therapy relieves the symptoms of acute pulmonary exacerbations, reduces the mortality, and improves the quality of life of CF patients (19). The usual therapy for CF patients colonized with *P. aeruginosa* involves the use of an aminoglycoside and/or a β-lactam. These antibacterial agents require frequent high-dose parenteral administration in order to achieve therapeutically effective concentrations in serum. Nephrotoxicity and ototoxicity are directly related to prolonged elevations of peak and trough concentrations of tobramycin in serum (15, 41, 42). Aerosolized antibiotic is also a current treatment that alleviates the need to produce high concentrations in serum, but there is no general agreement about its efficacy (21, 45). Despite the use of aggressive antibiotic therapy in the treatment of *Pseudomonas* infection in CF patients (23), eradication of the organism is rarely achieved at present (23, 37, 43). It has been suggested that biofilm formation by mucoid variants of *P. aeruginosa* in the lungs of CF patients may be an important cause of infection persistence (10, 28, 33).

Previous studies have shown that drug incorporation into liposomes can result in sustained local concentrations of antimicrobial agents (16, 39, 40). In a previous work performed with an animal model of chronic pulmonary infection with *P. aeruginosa*, we demonstrated that the intratracheal administration of rigid liposome-entrapped tobramycin increased markedly the residence time of antibiotic in the lungs and reduced systemic drug absorption without improving the bactericidal efficacy of the encapsulated drug (36). To promote more-efficient liposome-bacterium interaction, we designed and tested liposomal formulations with varied characteristics regarding membrane fluidity and electric charge. Fluid liposome, predominantly composed of dipalmytoyl phosphatidylcholine (DPPC) with the addition of a charged phospholipid (dimyristoyl phosphatidylglycerol [DMPG] [10:1 to 15:1 molar ratio]), showed modulated release of tobramycin in vitro while maintaining long-term pulmonary persistence of the drug (7).

The present study was undertaken to test the bactericidal efficacy of fluid liposome-entrapped tobramycin compared with those of rigid liposomes and free antibiotic in an animal model of chronic pulmonary infection with a mucoid strain of *P. aeruginosa*. To our current knowledge, this is the first time that chronic pulmonary infection caused by *P. aeruginosa* has been cured by liposome-encapsulated antibiotic.

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MATERIALS AND METHODS

Bacterial strain. A clinical isolate of *P. aeruginosa*, PA 508, was used throughout this study. PA 508 is a stable mucoid strain isolated from the sputum of a patient with CF (at Sainte-Justine Hospital, Montreal, Québec, Canada). The MIC of tobramycin for PA 508 is 1 μg/ml. Bacteria were stored at −70°C in brain heart infusion broth supplemented with 10% glycerol. For the experimentation, an 18-h culture of this organism in Proteose Peptone broth (Difco Laboratories, Detroit, Mich.) was used.

Liposomes. Liposomes were prepared with synthetic phospholipids (Avanti Polar Lipids, Inc., Birmingham, Ala). The first formulation consisted of 10:1 and 15:1 molar ratios of DPPC, a noncharged phospholipid, and DMPG, a negatively charged lipid. The second formulation was composed of disteroyl phosphatidylcholine (DSPC) and dimyristoyl phosphatidylcholine (DMPC), two noncharged lipids, at a 15:1 molar ratio. Liposomes were prepared by the dehydration-rehydration vesicle method (25, 27) with modifications. Briefly, appropriate amounts of lipid mixture were dissolved in chloroform in a round-bottom flask and dried to a lipid film by rotoevaporation (Bucci Rotavapor-KRvr 65/45) at 65°C under vaccuum (Bucci 168-Vacuum/Distillation Controller). The lipids were then redissolved 1:20 in phosphate-buffered saline (PBS) and lyophilized (Virtis Genesis 12EL Console freeze dryer; Canberra Packard, Toronto, Ontario, Canada) in vials at 4°C. Vials were kept at −70°C until use. After rehydration with tobramycin (Eli Lilly, Toronto, Ontario, Canada), liposomes were filtered in an extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia, Canada) charged successively with polycarbonate membranes with pore sizes of 1, 0.6, and 0.4 μm. Control liposomes were prepared similarly, but PBS was used instead of the antibiotic. The quantification of tobramycin in liposomes was performed by high-performance liquid chromatography (HPLC) as described below.

Experimental infection and antibiotic treatment. Thirty-five adult Sprague-Dawley rats weighing between 175 and 225 g (Charles River, Saint Constant, Québec, Canada) were used for this study. Animals were housed in groups of three and allowed free access to food and water. Chronic respiratory tract infection caused by *P. aeruginosa* PA 508 was induced as described previously (36) with slight modifications. Briefly, the bacterial strain was grown overnight at 37°C in Proteose Peptone broth (Difco Laboratories). Bacteria were then incorporated into agar beads, and a suspension containing 5×10^5 CFU/100 μl was administered intratracheally to anesthetized animals as previously described (36). Three days after the inoculation of bacteria coated with agar beads, animals received antibiotic treatments. For the first experiment, rats received three doses at 16-h intervals of either 0.1 ml of free tobramycin (600 μg) or 0.1 ml of liposome-encapsulated tobramycin (600 μg) by intratracheal instillation as described for the infection. Infected control animals were not treated or were treated with PBS or with liposome-entrapped PBS. For the second experiment, rats were treated twice with 240 μg of free or encapsulated tobramycin and the controls described above were used. Sixteen hours after the last treatment, animals were anesthetized and exsanguinated. The kidneys and the lungs were removed aseptically and homogenized in cold sterile PBS (40% [wt/vol]) for 30 s with a Polytron Homogenizer (Kinematica, Lucerne, Switzerland). The homogenizer was rinsed and flamed between samples. A 100-μl volume of homogenized lung tissue was immediately used for serial dilutions in PBS, and appropriate dilutions were plated and cultured in triplicate on Proteose Peptone no. 2 agar (Difco Laboratories). After an overnight incubation, the number of CFU was determined. *P. aeruginosa* colonies were tested on C-390 medium agar (9) to confirm the identity of the counted colonies. Samples (100 μl) of homogenized tissues were conserved at −70°C in a methanol extraction solution before HPLC analysis. The remainder of the tissue homogenates was conserved at −70°C.

HPLC analysis. Tobramycin concentrations were determined by using an HPLC system with UV detection as previously described (3–5). The HPLC system was composed of a pump, a system controller (Varian 5000), a simple 10-μl loop injector (Reodyne 7125), a UV spectrophotometric detector (Varian 2550), and a recorder (Spectra-Physics SP 4290). The separation was carried out on a Zorbax SB-C18 column (4.6 by 25 cm [inside diameter]; particle size, 4 μm; Du Pont Co.). The mobile phase was acetonitrile-methanol (MeOH)-potassium phosphate buffer (10 mM) (65:10:25 [vol/vol/vol] pH 3.5), and the flow rate was 1.3 ml/min. The column effluent was monitored at 350 nm and at 0.005 absorbance units full-scale. Chromatography was performed at room temperature.

The procedure for the determination of tobramycin concentrations was based on derivatization of the tobramycin with 1-fluoro-2,4-dinitrobenzene. Tobramycin contained in homogenized tissue samples and in liposomes was extracted as follows. A 100-μl portion of liposomes or homogenized tissue was added to 1 ml of MeOH, and the mixture was vortexed for 1 min and incubated at 65°C for 30 min. A 900-μl portion of PBS was then added, and the mixture was vortexed for 1 min and centrifuged at 5,000 × *g* for 20 min at 4°C. A 170-μl volume of supernatant was then transferred to a vial containing 90 μl of 1-fluoro-2,4-dinitrobenzene prepared in MeOH (180 g/liter), 60 μl of 0.1 M borate buffer (pH 9.3), and 670 μl of MeOH. Vials were sealed, vortexed, and heated for 30 min at 85°C and then cooled at room temperature. For liposome-encapsulated tobramycin analysis, standard curves were constructed in PBS with known amounts of tobramycin and processed as described above. Other standard curves were constructed with known amounts of tobramycin in homogenized control lung and kidney tissues and treated as described above. The limits of detectability for tobramycin in PBS and in lung and kidney tissues were 0.2 μg/ml, 0.3 μg/mg, and 0.5 μg/mg, respectively. Correlation coefficients of 0.99% were observed for the chromatographic peak areas and the tobramycin concentrations between 0.2 and 125 μg/ml of PBS, between 0.3 and 125 μg/mg of lung tissue, and between 0.5 and 125 μg/mg of kidney tissue samples. In PBS assays, the coefficients of variation at concentrations of tobramycin from 0.2 to 125 μg/ml ranged between 0.6 and 1.2%. For lung and kidney samples, the coefficients of variation at concentrations from 0.3 and 0.5 to 125 μg/mg ranged between 0.3 and 7.6% and 1.7 and 7.4%, respectively. Three sets of five quality control PBS samples at tobramycin concentrations of 3.9 and 125 μg/ml were analyzed to determine the intraday and interday variabilities of the assay; the coefficients of variation were of 0.01 and 0.1%, respectively, for intraday measurements and 0.1 and 1.2%, respectively, for interday measurements.

Microbiological assay. To measure the quantity of active tobramycin in tissues, tissue samples were concentrated as follows: 1.4-ml samples of homogenized lungs or kidneys were lyophilized (Virtis Genesis 12EL Console freeze dryer; Canberra Packard) and rehydrated with 150 μl of sterile PBS. The presence of active tobramycin was detected by a microbiological assay as previously described (36). Briefly, 25 μl of tissue samples and standards in duplicate were placed into agar wells on a glass plate and incubated overnight (24 h) at 37°C under 5% CO_2. Duplicate inhibition zone diameters were averaged and compared with a series of standards. Standard curves were made with known quantities of free tobramycin added to control homogenized lungs or kidneys. The concentrations of unknown samples were obtained by extrapolation from the zones of inhibition of standards by linear regression analysis of standards. The limits of detection for tobramycin in PBS and tissues were 3.9 μg/ml and 7.8 μg/mg, respectively. The correlation coefficients in PBS and tissues were 0.97 and 0.99, respectively, while coefficients of variation for intraday and interday measurements were ≤10%.

Data analysis. Bacterial counts were expressed as means ± standard errors of the means obtained from at least three plates per dilution. All computer analysis were performed with a linear regression system using Microsoft EXCEL version 5.0.

RESULTS

Following the demonstration that fluid, negatively charged liposomes prepared with DPPC-DMPG showed both modulated release in vitro and long-term pulmonary retention of tobramycin (unpublished results), two independent experiments were performed to measure the anti-*P. aeruginosa* bactericidal efficacy of encapsulated tobramycin. For the first experiment, tobramycin was encapsulated in two formulations: a formulation made of DPPC-DMPG at a 10:1 molar ratio, presenting a global phase transition temperature (Tc) of 29.5°C, and a formulation made of DSPC-DMPC at a 15:1 molar ratio, presenting a global Tc of 42.0°C. As controls, infected rats were treated with PBS, free tobramycin, and DPPC-DMPG and DSPC-DMPC liposome-encapsulated PBS. A 0.1-ml volume of liposomes containing 600 μg of tobramycin as determined by HPLC analysis was administered three times at 16-h intervals. For the second experiment, the formulation composed of DPPC-DMPG at a molar ratio of 15:1 was administered in two treatments of 240 μg of tobramycin at 16-h intervals. This latter formulation was characterized by a global Tc of 33°C.

An effective bactericidal activity against *P. aeruginosa* was observed in the five rats treated with encapsulated tobramycin with the formulation DPPC-DMPG at a molar ratio of 10:1 (Table 1). Indeed, the number of CFU (0 to 8) detected on the plates spread in triplicate with undiluted lung samples was far lower than the minimal CFU count (i.e., 30 CFU) considered statistically significant (26). Conversely, the use of encapsulated tobramycin in the formulation DSPC-DMPC did not show any improvement of bactericidal effect compared with the use of PBS, liposome-encapsulated PBS, or free tobramycin. The number of CFU enumerated following the treatment with tobramycin encapsulated in the DSPC-DMPC formulation preparation was in the range obtained with negative controls: 1.4×10^6 to 2.3×10^7 CFU per pair of lungs (Table 1).

The results of the second experiment showed that lower doses of encapsulated tobramycin in the DPPC-DMPG formulation (at a 15:1 molar ratio) were still very effective against *P.*

TABLE 1. Bactericidal effect of liposome-encapsulated tobramycin on *P. aeruginosa* in an animal model of chronic pulmonary infection

Rat[a]	CFU/pair of lungs with:					
	PBS only	Liposome-PBS[b]	Liposome-tobramycin[b]	Liposome-PBS[c]	Liposome-tobramycin[c]	Free tobramycin
1	1.8×10^6	3.8×10^6	0	1.9×10^7	1.5×10^6	2.0×10^5
2	4.9×10^5	6.0×10^7	4	1.4×10^7	4.3×10^6	4.2×10^5
3			0	2.6×10^7	1.5×10^6	1.8×10^7
4			4		1.7×10^6	1.4×10^6
5			8		1.9×10^5	4.2×10^7
Avg	1.1×10^6	2.3×10^7	—[d]	2.0×10^7	1.8×10^6	1.2×10^7

[a] Rats received three tobramycin treatments of 600 μg at 16 h intervals.
[b] Liposome composition, DPPC-DMPG (10:1 molar ratio).
[c] Liposome composition, DSPC-DMPC (15:1 molar ratio).
[d] —, those CFU numbers compiled on triplicated plates were far lower than the minimal CFU count (i.e., 30 CFU) considered statistically significant in accordance with reference 26. Therefore, these values cannot be considered significant.

aeruginosa infection. The administration of only two 240-μg doses of tobramycin instead of three 600-μg doses as previously used for the first experiment showed a comparable bactericidal effect. Almost complete eradication of the mucoid form of *P. aeruginosa* was observed. The number of CFU detected on the plates spreaded in triplicate with undiluted lung samples varied between 0 and 3 CFU. Such CFU numbers are far lower than the minimal CFU count (i.e., 30 CFU) considered statistically significant (26). Otherwise, $\geq 10^8$ CFU per pair of lungs were enumerated for control rats treated with PBS or with PBS encapsulated in the DPPC-DMPG formulation (Table 2).

Sixteen hours after the last treatment, animals were sacrificed and tobramycin in the lungs and kidneys was quantitated by HPLC and microbiological assays. HPLC analysis showed high concentrations of tobramycin (≥23 μg/mg of tissue) in the lungs after treatments with liposome-encapsulated tobramycin or free tobramycin (Fig. 1). Determination of tobramycin levels in the kidneys by HPLC following administration of liposome-encapsulated tobramycin gave low values (≤0.87 μg/mg of tissue), whereas 5.31 μg of tobramycin per mg of tissue was detected in the kidneys following the administration of free antibiotic (Fig. 1). The microbiological assay used to control for whether the antibiotic detected by HPLC was still active confirmed the activity of the whole antibiotic detected by HPLC in the lungs following its administration in the encapsulated form (Fig. 2). As shown in Fig. 1 and 2, the quantities of antibiotic detected by both methods gave approximately the same results. However, important differences between antibiotic determination performed by HPLC and that performed by microbiological assay following the administration of free antibiotic were observed: analysis showed that 23.3 ± 19.51 μg of tobramycin per mg of lung tissue was detected by HPLC analysis compared with traces detected by the microbiological assay (Fig. 1 and 2). Tissue samples studied by microbiological assay showed a pH of 6.8, compatible with normal activity of tobramycin. The determination of tobramycin levels in the kidneys by microbiological assay after the administration of encapsulated antibiotic allowed the detection of nonquantifiable traces of antibiotic, while the HPLC analysis gave values between 0.59 and 0.87 μg/mg of tissue.

TABLE 2. Bactericidal effect of liposome-encapsulated tobramycin on *P. aeruginosa* in an animal model of chronic pulmonary infection

Rat[a]	CFU/pair of lungs with:			
	PBS only	Liposome-PBS[b]	Liposome-tobramycin[b]	Free tobramycin
1	1.8×10^8	1.0×10^8	0	1.6×10^6
2	3.1×10^7	1.5×10^8	3	1.6×10^5
3	1.4×10^8	1.1×10^8	3	1.5×10^6
Avg	1.0×10^8	1.2×10^8	—[c]	1.0×10^6

[a] Rats received two tobramycin treatments of 240 μg at 16-h intervals.
[b] Liposome composition, DPPC-DMPG (15:1 molar ratio).
[c] See Table 1, footnote *d*.

DISCUSSION

The liposomal formulation tested herein was previously selected because of its (i) encapsulation capacity, (ii) fluidity allowing modulated release of antibiotic and possible interaction with the bacterial outer membrane, (iii) pulmonary persistence, and (iv) negative charge (7). The results presented here demonstrate for the first time that chronic pulmonary infection caused by mucoid *P. aeruginosa* can be cured by in situ administration of liposome-encapsulated tobramycin. Three treatments with 600 μg of tobramycin or two treatments with 240 μg of tobramycin encapsulated in a liposomal formulation composed of DPPC and DMPG at a molar ratio of 10:1 or 15:1 allowed almost complete eradication of mucoid *P. aeruginosa*. More than 10^6 CFU per pair of lungs were enu-

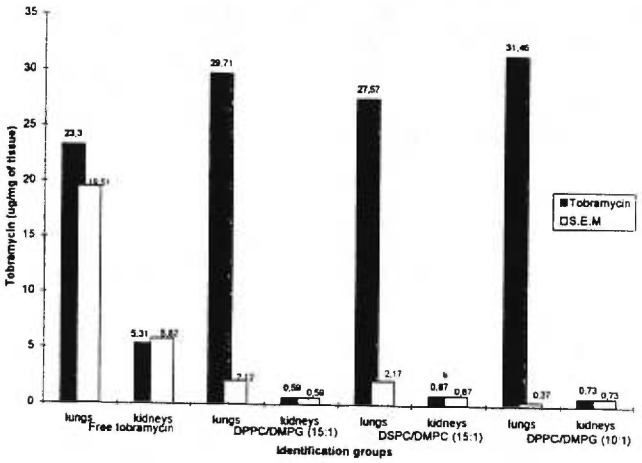

FIG. 1. Measurement of tobramycin levels in lung and kidney homogenates of rats chronically infected with *P. aeruginosa* by HPLC. Tissues were removed at 16 h after the last antibiotic treatment. S.E.M., standard error of the mean. Values in parentheses correspond to molar ratios.

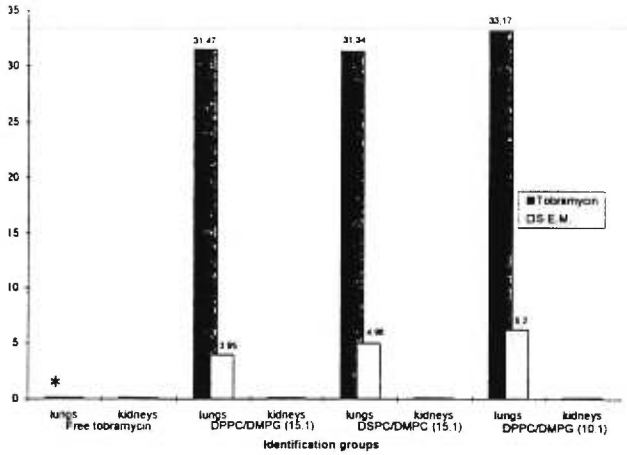

FIG. 2. Measurement of tobramycin levels in lung and kidney homogenates he rats described in the legend to Fig. 1 by microbiological assay. S.E.M., ıdard error of the mean. The asterisk refers to the five lowest bars of the ɔh and means that only traces of tobramycin were detected by the microbi-gical assay. Values in parentheses correspond to molar ratios.

:rated after treatments with free tobramycin or with lipo-ne-encapsulated PBS. Moreover, other liposomal formula-ns tested (DSPC-DMPC and DSPC-DMPG) did not show y bactericidal activity as demonstrated herein and in a pre-us work (36).

These results indicate that the lipid composition of liposo-l formulations has a preponderant influence on the bacte-idal efficacy of encapsulated drugs. The high-level bacteri-al efficacy of the formulations consisting of DPPC and MPG at molar ratios of 10:1 and 15:1 seems dependent marily on their levels of fluidity, due, respectively, to Tcs of 5 and 33°C. Conversely, liposomal formulations composed DSPC and DMPG (molar ratio of 10:1) and DSPC and MPC (molar ratio of 15:1), whose Tcs were, respectively, 40 d 44°C, presented a rigidity that seems incompatible with ɔdulated release of antibiotic and/or bacterium-liposome in-action. In addition, the negative charge of the fluid lipo-nes may favor their reactivity. It has been demonstrated that h an equivalent Tc, the negative charge tends to improve : physical stability of liposomes by reducing the rate of ag-:gation (11, 20). Nevertheless, a negative charge cannot by :lf promote bactericidal efficacy, since such activity was not served with the negatively charged formulation DSPC-MPG (36). Thus, it may be hypothesized that the marked .cacy of the formulation DPPC-DMPG can be explained by improved passage of encapsulated antibiotic through the ɔpolysaccharide barrier and/or by an increase in penetration the antibiotic through the bacterial outer membrane, possi-by a mechanism of fusion. Whereas fluidity of liposomes :ms to promote interaction between liposomes and bacterial ls, liposomal rigidity seems unable to induce such interac-ns.

Another interest of this work lies in the fact that the lipo-nal formulation DPPC-DMPG succeeded in curing a ·onic infection caused by a mucoid variant of *P. aeruginosa*. ıcoid variants are particularly resistant to conventional an-iotherapy, and they are the main cause of morbidity and ɔrtality among patients with CF. Therefore, the efficacy of ɔsome-encapsulated antibiotics against *P. aeruginosa* is not ɔrecedent. Researchers have demonstrated, although at a ser level, with keratitis and surgical wounds, improvement of bactericidal activity after topical administration of liposome-encapsulated antibiotics specific to *P. aeruginosa* (2, 39, 40). Unfortunately, information concerning the lipid compositions and Tcs of their liposomes are lacking.

The fact that HPLC analysis and microbiological assays measured the same level of antibiotic in the lungs after the administration of encapsulated tobramycin shows that the tobramycin detected under those conditions was still active. However, only traces of tobramycin were detected in the lungs by microbiological assay after administration of free antibiotic. The same samples analyzed by HPLC gave a concentration of 23 ± 19.51 μg of tobramycin per mg of lung tissue, showing that there was antibiotic but that it was in an inactive form. Previous studies have demonstrated the inactivation of aminoglycosides in CF sputum in part by binding to purulent sediment rather than degradation (8, 29, 31). Other studies have demonstrated that the absence of antibacterial activity of aminoglycosides in CF secretions could be related to large amounts of DNA present in secretions and to interactions with ribosomes (12, 38). Important variations between kidney samples suggest pronounced individual characteristics concerning kidney retention of antibiotics. HPLC analysis and microbiological assay showed different values concerning levels of antibiotic measured in the kidneys after administration of free tobramycin. Whereas 5.31 μg of tobramycin per mg of kidney tissue was detected by HPLC analysis, only traces were found by microbiological assays. This suggests that the antibiotic detected by HPLC in the kidneys was not active. The difference, since low levels were measured, may also be due to the lower-level sensitivity of the microbiological test. Nevertheless, HPLC analysis showed that 0.59 to 0.87 μg of tobramycin per mg of tissue was present in the kidneys after administration of encapsulated antibiotic compared with 5.31 μg/mg of tissue with free antibiotic. This may suggest reduction of renal toxicity with administration of encapsulated antibiotics.

The demonstration of the bactericidal efficacy of our liposomal formulation may not be limited to *P. aeruginosa* strains that present both in vitro susceptibility and in vivo resistance to antibiotics. If intrinsic drug resistance, mainly due to the outer membrane diffusion barrier, can be reversed by our liposomal formulation, it is probable that this formulation could also be efficient against strains with acquired resistance. Evidence shows that the low-level permeability of the outer membrane also plays an important role in acquired antibiotic resistance. Low-level permeability would act in a synergistic way with antibiotic-degrading enzymes (6, 17, 34, 35). The target access index would then be proportional to the permeability coefficient of the drug and inversely proportional to the drug inactivation rate in the periplasm (35, 44). In the same way, it would be very surprising if the liposomal formulation developed here did not have improved bactericidal activity against other gram-negative bacteria related to *P. aeruginosa*, like *Xanthophilia maltophilia* and *Burkholderia cepacia*.

In conclusion, the liposomal formulation developed here could greatly improve the treatment of chronic pulmonary infection in CF patients. Works are in progress with larger groups of animals to adapt a nebulization method to the in situ administration of the liposomes described herein.

ACKNOWLEDGMENT

This study was partially supported by a grant from the Fonds de la Recherche en Santé du Québec.

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