J. Pharm. Pharmacol. 1988, 40: 166–170
Received August 7, 1987

Evaluation of Reconstituted Sendai Virus Envelopes as Intra-articular Drug Vectors: Effects on Normal and Experimentally Arthritic Rabbit Knee Joints

RUPERT T. EARL, *IAN M. HUNNEYBALL, †E. ELLEN BILLETT AND R. JOHN MAYER

*Department of Biochemistry, The Queen's Medical Centre, University of Nottingham, Clifton Boulevard, Nottingham NG7 2UH, * The Research Department, The Boots Company PLC, Nottingham NG2 3AA, and † Department of Life Sciences, Trent Polytechnic, Clifton, Nottingham NG11 8NS, UK*

Abstract—Fusogenic vesicles reconstituted from the envelopes of Sendai virus particles were injected into rabbit knee joints (both normal and experimentally arthritic) to evaluate the in-vivo biocompatibility of these putative drug carriers. The reconstituted Sendai virus envelopes (RSVE) were >80% retained within the arthritic knee joints after 24 h and studies with ^{125}I- and fluoroscein-labelled RSVE both showed association of the vesicles with the synovia of arthritic and healthy joints. However, RSVE were found to cause inflammation after intra-articular injection, as judged by joint swelling and histological assessment, and these effects were exacerbated by successive administrations. RSVE-entrapped methotrexate, whether free or conjugated to human serum albumin, was ineffective in preventing the irritancy of RSVE or in reducing the chronic inflammation in joints affected by an experimentally induced arthritis.

In rheumatoid arthritis intra-articular injections are used commonly to localize the delivery of drugs to the diseased joint(s). Nevertheless, the usefulness of this mode of treatment is restricted by efflux of injected drug from the joint into the circulation, with consequent systemic actions (Bird et al 1979). Attempts to improve intra-articular drug retention have included incorporation of injected drug into supramolecular or polymeric carriers which are taken up by both resident inflammatory cells and those infiltrating from the circulation into the synovium. For instance, liposomal encapsulation has been used to improve the joint retention of methotrexate and cortisol palmitate (Foong & Green 1983a,b; Dingle et al 1978). More recently, cross-linked albumin microspheres have also been proposed as intra-articular drug carriers (Ratcliffe et al 1984). The success of such approaches depends firstly on the fact that the carrier will remain intact whilst it persists extracellularly, and secondly that drug will not leak passively from the carrier to a significant extent. But it also depends fundamentally on the internalization of carrier by the inflammatory cells either by phagocytosis or endocytosis. Consequently, the ultimate destination for internalized carrier-borne drugs is lysosomes or intracellular vacuoles (Straubinger et al 1983), which are topographically equivalent to the outside of the cell. Unless escape from the endocytic pathway can be engineered (Connor et al 1984), only membrane-permeant, small molecules will escape significantly from lysosomes into the cytosol (Straubinger et al 1983; Lloyd & Forster 1986).

An alternative strategy is to use a vesicular carrier which can microinject encapsulated drug molecules directly into the cytoplasm of target cells through a fusion event at the plasma membrane. Membrane vesicles which are intrinsically fusogenic at neutral pH could be used for this purpose. The reconstituted envelope membranes of paramyxoviruses (e.g. Sendai virus) are such a microinjection vector. Reconstituted Sendai virus envelopes (RSVE) are unilamellar vesicles which incorporate two membrane glycoproteins and enclose an intravesicular aqueous space (Earl et al 1987). Attachment of RSVE to cells is mediated by the haemagglutinin-neuraminidase (HN) protein, and fusion of RSVE with the plasma membrane requires both the HN and the fusion (F) protein functions (Gitman & Loyter 1984; Martinez et al 1986).

Recently the use of RSVE, or of proteoliposomes incorporating the Sendai fusogen proteins, as possible drug vectors in-vivo has been proposed (Martinez et al 1986; Loyter et al 1986), but to our knowledge has not previously been attempted. In this study, we evaluated the feasibility of using RSVE as intra-articular drug carriers in experimental arthritis. In principle RSVE should microinject encapsulated (drug) molecules into the cytoplasm of cells in-vivo as has been demonstrated for the fusion-mediated delivery of RSVE-entrapped molecules into animal cells in-vitro (Vainstein et al 1984; Gitman et al 1985; Arad et al 1986). For this reason, RSVE offer advantages over other supramolecular drug carriers. Firstly, drug molecules carried in RSVE are less likely to become sequestered in lysosomes or intracellular vacuoles. Secondly, as drug delivery does not require phagocytosis of the carrier it may be possible to modulate the activities of cells which have a low phagocytic index, rather than cells such as macrophages which would otherwise outcompete less phagocytic cells for the available carrier. For instance, in the synovial membrane there are type A, B and C cells which differ markedly in their phagocytic index (Barland et al 1962) and fibroblasts, plasma cells, macrophages and T-lymphocytes also invade inflamed synovia.

Correspondence to: Rupert T. Earl, Dept of Biochemistry, The Queen's Medical Centre, University of Nottingham, Clifton Boulevard, Nottingham NG7 2UH, UK.

Materials and Methods

Preparation of reconstituted Sendai virus envelopes (RSVE)
Sendai virus was propagated as described by Earl et al (1987). RSVE were prepared from Sendai virus particles by the method of Vainstein et al (1984). The reconstituted vesicles were harvested by Sephadex G25M gel filtration eluted with ice-cold, pH 7·4 buffer containing 160 mM NaCl, 20 mM Tricine-NaOH (Buffer 1).

Labelling procedures
RSVE labelled with fluorescein isothiocyanate (FITC) were prepared as described by Chejanovsky et al (1984). Radiolabelling of RSVE with ^{125}I was carried out as described in Earl et al (1987).

Encapsulation of drugs in RSVE
Drug molecules to be encapsulated in RSVE were added to detergent-solubilized Sendai virus envelopes immediately before reconstitution of the vesicles, as described in Vainstein et al (1984). Methotrexate was added to the solubilized envelopes obtained from 7–8 mg of virus to a final concentration of 2·5 mM. Methotrexate conjugated to human serum albumin (HSA) protein (molar substitution ratio 25:1) as described by Garnett et al (1983) was added to a concentration of 1·33 mg mL^{-1} with respect to the HSA moiety; control RSVE were constructed from a reconstitution mixture containing HSA to a concentration of 1·33 mg mL^{-1}. RSVE containing methotrexate were separated from uncaptured free drug by gel filtration on Sephadex G25M (as above). RSVE containing methotrexate conjugated to HSA, or HSA alone, were separated from uncaptured conjugate or HSA, respectively, by centrifugation in a microfuge (6000 *g* for 5 min). The RSVE pellets were resuspended in ice-cold Buffer 1, washed by recentrifugation and finally resuspended as before.

The specific activities of RSVE vesicles reconstituted in the presence of the drugs were (approx.): methotrexate = 3 ng μg^{-1}, and methotrexate as a conjugate with HSA = 7·5 ng μg^{-1} of RSVE protein.

Cell culture and fusion with RSVE
Mouse macrophages obtained by peritoneal lavage were cultured on glass coverslips in Medium 199 containing 10% (v/v) swine serum, glutamine (0·1 mg mL^{-1}), benzylpenicillin (60 $\mu g\ mL^{-1}$) and streptomycin (100 $\mu g\ mL^{-1}$). Macrophage-RSVE fusion was performed as described in Earl et al (1987) for hepatoma cell monolayers, but using Buffer 1 containing 15 μM $LaCl_3$ as the fusion buffer.

Induction of arthritis in rabbits
Antigen-induced arthritis was established unilaterally in the right knee joint of age- and weight-matched, young, adult New Zealand white rabbits as described previously (Hunneyball 1981).

Assessment of arthritis
The inflammatory reaction within the rabbit knee joints was monitored routinely by measurement of joint diameter using engineering callipers.

At the end of the experiment, the animals were killed with sodium pentobarbitone, the knee joints opened and the macroscopic changes noted. The infrapatellar fold containing synovial membrane and underlying tissue was removed, fixed in buffered formalin, dehydrated in ethanol and embedded in paraffin wax. Sections 5 μm thick were cut and stained with haematoxylin/eosin or Van Gieson's stain. For the fluorescence studies, the tissues were snap frozen in a dry ice:isopentane mixture and frozen sections taken.

Intra-articular administration of RSVE
Each animal received 150 μg of RSVE (vesicle protein) suspended in 0·3 mL of Buffer 1 injected into the right (arthritic) and/or left (healthy control) knee joint cavity. In short term studies, rabbits were killed 24–48 h after receiving RSVE and the infrapatellar folds containing the synovial membrane and submembranous adipose tissue were removed for histological examination. For longer term studies, successive injections of RSVE (as above) were made on days 0, 9 and 14 and joint diameters were measured at intervals. The rabbits were killed on day 15 for histological examination of synovial tissues. Control animals received intra-articular injections of 0·3 mL of Buffer 1 only.

Results

The RSVE used were unilamellar vesicles (200–300 nm in diameter, determined by electron microscopy) which incorporated the Sendai viral haemagglutinin-neuraminidase (HN) and fusion (F) proteins (Earl et al 1987). The fusogenic activity of RSVE with animal cells in-vitro was demonstrated by fluorescence photomicroscopy of mouse peritoneal macrophages cultured on glass coverslips following fusion with a suspension of fluorescein-labelled RSVE vesicles (not shown).

Table 1. Distribution of ^{125}I radioactivity 24 h after intra-articular injection of [^{125}I]RSVE into rabbit arthritic knee joints.

Organ or tissue	% of total counts min^{-1} recovered (mean)
Tissue of injected joint	
Synovium	12·96
Articular cartilage	0·01
Ligamentum patellae	0·51
Menisci	1·01
Patella	0·76
Flexor digitorum	0·41
Synovial fluid	65·59
Other tissues	
Blood*	4·88
Thyroid	13·84
Liver	+
Kidney	+
Spleen	+
Lung	+
Skeletal muscle	+

Three animals each received 150 μg of ^{125}I-labelled RSVE (protein) in 0·3 mL sterile Buffer 1 into the right (arthritic) knee joint.
* Estimated total blood volume = 250 mL; + radioactivity < twice background. Total recovered radioactivity, counts min^{-1} $animal^{-1}$ ± s.e.m. = 314 930 ± 100 520.

Table 2. Inflammatory changes in the healthy control joints of rabbits following three intra-articular injections of RSVE preparations

	Mean macroscopic scores			Mean histological scores[3]			
Treatment group	Synovium[1]	Fluid[2]	Total	Hyperplasia + villi	Cellular Infiltration	Fibrosis + Blood vessels	Total
Buffer 1	1·2	1·6	2·8	0·6	0·8	0·5	1·9
RSVE control (entrapping HSA)	2·4	2·4	4·8	1·8	2·5	1·5	5·8
RSVE entrapping methotrexate-HSA Conjugate	3·2	3·2	6·4	1·8	2·2	1·5	5·5
RSVE entrapping methotrexate	2·4	3·2	5·6	1·8	1·8	1·6	5·2

Mean macroscopic scores ([1]) synovial discolouration, and ([2]) amount of synovial fluid, both graded on 0–4 scale. Mean histological scores ([3]): each characteristic scored on 0–4 scale; observations based on two sections through each intrapatellar fat pad (5 animals per group).

The fate of fluorescein-labelled RSVE vesicles injected into the healthy knee joints of rabbits was studied histologically 48 h after injection. In post-mortem synovial sections, fluorescence was associated with the cells of the synovial membrane and also with isolated cells (probably macrophages) lying deeper within the underlying tissues. The localization of fluorescently labelled RSVE injected into arthritic knee joints was difficult to identify due to the disorganized histology of these chronically inflamed synovia.

Since the ultimate aim of this work is to improve the therapeutic management of arthritic joints, the localization of RSVE within the arthritic knee joints of rabbits was investigated further using radioiodinated RSVE vesicles. Three animals were killed 24 h after each had received a single intra-articular injection of ^{125}I-labelled RSVE and the distribution of radioactivity within the joint and in other tissues was measured. Most (80%) of the radioactivity was retained in the joints after 24 h (Table 1) and was associated predominantly with the synovia and the synovial fluid. With the exception of thyroid tissue (which actively accumulated ^{125}I released by dehalogenation of iodotyrosine and iodohistidine from degraded RSVE from the blood) only very small amounts of radioactivity were found in extra-articular tissues and organs.

The potential irritancy of RSVE injected into rabbit knee joints was investigated by histological examination of the synovia. In synovia from the *normal* knee joints of rabbits injected 48 h previously with fluorescein-labelled RSVE evidence of acute localized irritancy was found. The histological changes included local synovial lining cell hyperplasia and villus formation in the synovial membrane, and connective tissue deposition, increased vascularity and cellular infiltration of the sub-membranous layers. To assess the rapidity of onset of these changes and to exclude the possibility that the FITC moiety itself caused the irritancy, unlabelled RSVE were injected into the normal knee joints of three further animals and after 24 h the joints were examined post mortem. In each case the synovium was slightly discoloured, although little or no synovial fluid could be recovered (unlike in severely inflamed joints). Histologically, there was evidence of slight, acute inflammatory changes of the type seen before, but these were in localized areas and were not representative of each synovium as a whole (2–3 sections examined per synovium in 4 joints). Furthermore sections from the synovium of a joint injected only with 0·3 mL Buffer 1 also displayed some localized inflammatory changes, suggesting that the changes observed previously were partially the consequence of physical trauma or sensitivity to the buffer vehicle. The changes seen following injection of fluorescein-labelled RSVE were unlikely to result, therefore, from sensitivity to the fluorescein moiety used in that study.

To investigate whether the changes seen in synovia were indeed the consequence of the irritancy of RSVE or were due to another factor (e.g. physical trauma) we carried out a study in which twenty rabbits each received intra-articular injections of either Buffer 1 or of an RSVE preparation in this vehicle. Fourteen days before the first RSVE injection, experimental arthritis was induced in one knee joint (right) of each animal. Each animal then received 150 μg of RSVE protein in 0·3 mL Buffer 1 (or 0·3 mL Buffer 1 alone), into both the left (normal) and right (arthritic) knee joints on day 0 and thereafter on days 9 and 14. The RSVE preparations used were (i) control RSVE vesicles entrapping HSA protein; (ii) RSVE vesicles entrapping methotrexate conjugated to HSA; and (iii) RSVE vesicles entrapping unconjugated methotrexate. The purpose of injecting RSVE containing either protein-conjugated or free methotrexate was to see whether this drug would be effective i) in preventing inflammatory changes resulting from the injection(s) and ii) in ameliorating the condition of established arthritic joints.

To monitor the effects of the various treatments, the diameter of each joint was measured at intervals (Fig. 1), and, on day 15, post-mortem macroscopic and histological changes in the joints were assessed (Table 2). The combined data strongly suggest that RSVE do produce inflammatory changes in the synovium of healthy joints, and that these changes were more severe than those seen after a single injection (Figs 1 and 2). Thus, although the increase in joint diameter following an injection of RSVE is apparently transient (lasting 48–72 h) the underlying histological changes are cumulative. Successive physical intrusions into a joint due to the intra-articular injection of Buffer 1 also produced slight inflammatory responses, but these were nowhere near as marked as seen with the RSVE preparation. The presence of methotrexate in RSVE, either free or conjugated to protein, was ineffective in reversing the irritancy of RSVE.

In knee joints affected by the experimentally induced arthritis the macroscopic scores for synovial discoloration

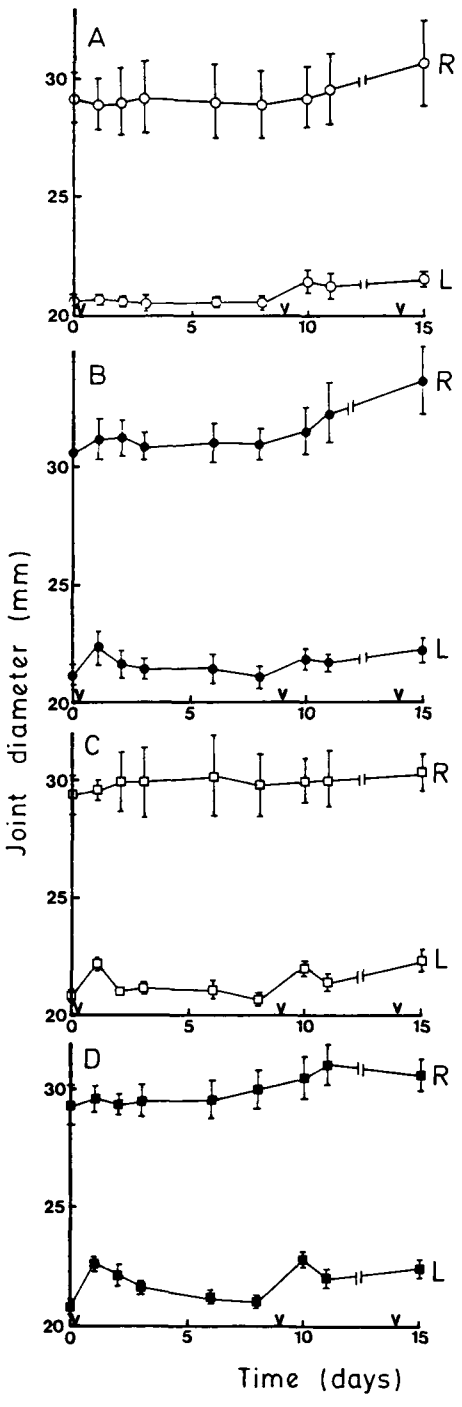

FIG. 1. Graphs showing changes in the diameters of left (control) and right (arthritic) knee joints in rabbits which had received (A) Buffer 1; (B) RSVE containing HSA; (C) RSVE containing methotrexate conjugated to HSA; (D) RSVE containing methotrexate. Each injection of RSVE was 150 μg of protein in 0·3 mL of Buffer 1; arrows indicate times of each injection.

and fluid exudation were the maximum possible in each case. Due to a lack of any obvious changes in the synovial histology in any of the treatment groups, no attempt was made to quantitate the histological status of arthritic joints. The presence of methotrexate in either form entrapped in the RSVE did not reduce the basal diameter of the swollen arthritic joints.

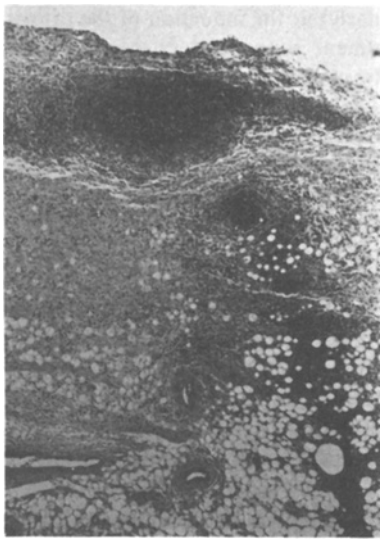

FIG. 2. Photomicrograph showing inflammatory changes in the synovium (magn. × 160) taken from a rabbit (left) knee joint after receiving 3 successive injections of 150 μg RSVE on Days 0, 9 and 14. The animal was killed 24 h after the final injection and the section shows massive cellular infiltration, fibrosis and the formation of lymphoid follicles.

Discussion

This study investigated the feasibility of using a novel drug vector, RSVE, as intra-articular drug carriers in the treatment of an experimental arthritis in rabbits. The data show that RSVE were largely retained (80%) in arthritic joints for at least 24 h with apparently little degradation. Both fluorescence- and radiolabelling of RSVE were used to show the association of the vesicles with synovial cells (13%), although most (65%) of the [^{125}I]RSVE were recovered in the synovial fluid. As RSVE are inherently fusogenic, it is unlikely that they persisted in the fluid as free entities. Rather it suggests that the RSVE had fused with the infiltrate of inflammatory cells which occurs in the synovial fluid of arthritic joints. The fusogenicity of the RSVE may account for the fact that both their retention in arthritic joints and their association with the synovium was considerably better than has been achieved with non-fusogenic liposomes. Foong & Green (1983a) found that only approximately 40% of multilamellar liposomes were retained in rabbit arthritic knee joints after 24 h, and < 1·5% were associated with the synovial membrane.

As intra-articularly administered RSVE associated efficiently both with the synovium and apparently also with cells in the synovial fluid, it is possible that these cells represent possible targets for pharmacological manipulation by RSVE-entrapped drugs. In this study methotrexate, which has been used successfully to treat rheumatoid arthritis (Weinstein et al 1985; Weinblatt et al 1985), was trapped in RSVE either as the free drug or conjugated to HSA. The improved retention of liposome-entrapped methotrexate in rabbit arthritic knee joints has been demonstrated before (Foong & Green 1983b). These workers found that 0·1 mg of liposomal methotrexate was as effective as 1·0 mg of free methotrexate in reducing joint swelling if administered

(intra-articularly) at the induction of the arthritis, but that neither treatment suppressed established arthritis. When RSVE vesicles containing either free methotrexate or methotrexate conjugated to HSA were injected into joints affected by an established arthritis in this study, no improvement in either histological or macroscopic inflammatory parameters was observed. The reason for this lack of success probably owes much to the fact that the RSVE vector itself produced inflammatory changes in the soft tissues of the joint. A possible explanation for the irritancy of the RSVE in-vivo may be the finding that fusion of RSVE with macrophages in-vitro stimulates the release of lysosomal enzymes (Earl & Hunneyball, unpublished observation).

Clearly the suitability of any drug carrier depends on its tissue acceptability in-vivo and the irritancy of RSVE vesicles in injected joints may compromise their usefulness as drug vectors. However, the changes elicited in the diameter of normal joints were transient and much less than those caused by the arthritis in the right joints. Indeed, the inflammatory changes (Fig. 2) were seen acutely following three injections in a period of only 2 weeks, and thus represent the worst possible situation.

In addition to the lack of success in ameliorating the established arthritis, methotrexate entrapped in RSVE vesicles did not diminish their irritancy in normal joints. It may be, therefore, that the amount and duration of action of the delivered drug was insufficient to achieve an observable effect. However, this does not preclude the possibility that RSVE may perform effectively as vectors for potent anti-inflammatory drugs, e.g. steroids, or agents which need to be restricted to precise targets in small quantities. These include polypeptide toxins (Gitman & Loyter 1985), and the radioisotopes used for the purpose of radiosynovectomy. In the latter case, the improved retention of ^{90}Y in rabbit arthritic knee joints by liposomal encapsulation has been described previously (Bard et al 1983).

Acknowledgements

We thank Dr M. Garnett of the Cancer Research Laboratories, University of Nottingham for the gift of methotrexate conjugated to HSA. We also thank Mrs Jayne Newham for preparing the mouse macrophages, and Mrs S. Sills for assisting with rabbit dissections and measurements. R.T.E. was supported by an S.E.R.C. CASE Award in conjunction with the Boots Company PLC, Nottingham, UK.

References

Arad, G., Hershkovitz, M., Panet, A., Loyter, A. (1986) Biochim. Biophys. Acta 859: 88–93

Bard, D. R., Knight, C. G., Page-Thomas, D. P. (1983) Clin. Exp. Rheumatology 1: 113–117

Barland, P., Novikoff, A. B., Kamerman, D. (1962) J. Cell. Biol. 14: 207–220

Bird, H. A., Ring, E. F. G., Bacon, P. A. (1979) Ann. Rheum. Dis. 38: 36–39

Chejanovsky, N., Beigel, M., Loyter, A. (1984) J. Virol. 49: 1009–1013

Connor, J., Yatvin, M. B., Huang, L. (1984) Proc. Natl. Acad. Sci. USA 81: 1715–1722

Dingle, J. T., Knight, C. G., Page-Thomas, P., Phillips, N. C., Shaw, I. H., Fildes, F. J. T., Oliver, J. E., Turner, E. M., Lowe, J. S. (1978) Nature (London) 271: 371–373

Earl, R. T., Billett, E. E., Hunneyball, J. M., Mayer, R. J. (1987) Biochem. J. 241: 801–807

Foong, W. C., Green, K. L. (1983a) Br. J. Pharmacol. 80 Suppl.: 522P

Foong, W. C., Green, K. L. (1983b) Ibid. 80 Suppl.: 692P

Garnett, M. C., Embleton, M. J., Jacobs, E., Baldwin, R. W. (1983) Int. J. Cancer 31: 661–670

Gitman, A. G., Loyter, A. (1984) J. Biol. Chem. 259: 9813–9820

Gitman, A. G., Graessmann, A., Loyter, A. (1985) Proc. Natl. Acad. Sci. USA 81: 7309–7313

Hunneyball, I. M. (1981) Agents Actions 11: 490–498

Lloyd, J. B., Forster, S. (1986) Trends Biol. Sci. 11: 365–368

Loyter, A., Gitman, A. G., Chejanovsky, N., Nussbaum, O. (1986) in: Celis, J. E., Graessmann, A., Loyter, A. (eds) Microinjection and Organelle Transplantation Techniques. Academic Press Inc., London, pp 179–197

Martinez, O., Kimura, J., Henry, C., Wolsy, L. (1986) Exp. Cell Res. 166: 180–190

Ratcliffe, J. H., Hunneyball, I. M., Smith, A., Wilson, C. G., Davis, S. S. (1984) J. Pharm. Pharmacol. 36: 431–436

Straubinger, R. M., Hong, K., Friend, D. S., Papahadjopoulos, D. (1983) Cell 32: 1069–1075

Vainstein, A., Hershkovitz, M., Israel, S., Rabin, S., Loyter, A. (1984) Biochim. Biophys. Acta 773: 181–188

Weinblatt, M. E., Coblyn, J. S., Fox, D. A., Fraser, P. A., Holdsworth, D. E., Glass, D. N., Trentham, D. E. (1985) N. Engl. J. Medicine 31: 818–822

Weinstein, A., Marlowe, S., Korn, J., Farouhar, F. (1985) Am. J. Medicine 79: 331–337