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Liposome encapsulated aurothiomalate reduces collagen-induced arthritis in DBA/1J mice

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Abstract

Collagen-induced arthritis (CIA) generated in rats or mice has long been a model system for the study of rheumatoid arthritis in humans. In particular, this system has been used to study the mechanisms and effects of anti-arthritic drugs in the treatment of the disease. Sodium aurothiomalate (ATM) is an agent often used to treat rheumatoid arthritis in humans; however, it possesses inherent toxicities which limits its usefulness. Liposome-encapsulated drugs are currently being developed to minimize the toxicities associated with a variety of potentially beneficial drugs. We have chosen to encapsulate ATM into small unilamellar vesicles (SUVs) to determine whether greater efficacy would be achieved in treating CIA with SUV ATM as compared to using the free drug. SUVs were prepared from hydrogenated egg phosphatidylcholine and cholesterol. These SUVs were very stable. Vesicles stored at 4°C lost only 0.09% of encapsulated ATM (SUV ATM) after 14 days and were able to reduce collagen-induced arthritis in these mice. Animals treated by i.m. injections of SUV ATM exhibited a 50% reduction in symptoms. More importantly, histological examination of knee joints of the affected animals verified that SUV ATM treatment prevented cellular infiltration of lymphocytes into the synovia of the collagen-sensitized mice. Conditioned media from spleen cell cultures was assayed for the presence of inflammatory lymphokines that might be affected by SUV ATM to account for the success in suppressing collagen-induced arthritis.

Keywords: Rheumatoid arthritis; Liposome; Sodium aurothiomalate; Collagen-induced arthritis

1. Introduction

Rheumatoid arthritis (RA), an autoimmune disease of unknown origin, is associated with inflammation of the joints and a variety of systemic problems [1,2]. In addition to joint erosion, it is not uncommon to find subcutaneous nodules, soft tissue swelling, muscle weakness, vasculitis, or metabolic bone disorders, such as osteoporosis [1]. Inflammation in the joints leads to pannus formation, an array of infiltrated lymphocytes, and fibrin in the joints. With time, the painful erosion of the cartilage in the joint takes place. This inflammatory response is mediated by the participation of synovial macrophages, $CD4^+$ and $CD8^+$ T-cells [3]. Work by Toyosaki suggests that antigens recognized by these infiltrating $CD4^+$ T-cells are exclusively on synovial cells

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[4,5]. Moreover, the presence of inflammatory lymphokines such as IL-1, TNF-α, IL-4 as well as TGF-β1 can be detected immunohistochemically in the synovium [5–12].

Rheumatoid arthritis has been subject to many therapies designed to suppress joint pain and inflammation. The failure to identify the etiology of this autoimmune disorder, makes the rational approach to effective drug therapy a fairly random process and less than curative. Anti-inflammatory drugs transiently reduce symptoms, yet the disease progresses over time. In addition, non-steroidal anti-inflammatory drugs induce gastric or hepatic toxicity [13]. The gold salts, among them aurothiomalate (ATM) and auranofin have long been used as anti-arthritic drugs and do retard the progressive destruction of cartilage. They are part of a group of disease-modifying drugs used to treat RA. Aurothiomalate (ATM) is a water-soluble thiogold derivative of malic acid and is used as parenteral drug. Lower doses of ATM (20 mg/month versus 40 mg/month) have also been used with some positive results [14]. Unfortunately, this gain in therapeutic efficacy elicited by ATM is accompanied by systemic toxicities, such as myelosuppression, hepatic toxicity, and dermatitis or stomatitis [15]. Patients must then cease this type of therapy. Other disease-modifying drugs, such as methotrexate [16,17], suffer from toxicities associated with anti-neoplastic drugs, which limits their long-term usefulness. These disease-modifying drugs appear to suppress the induction of TNF-α and IL-1 [18,19].

The objective here has been to use a well-known mouse model of rheumatoid arthritis [20,21,37] collagen-induced arthritis (CIA), to evaluate the potential of liposome-encapsulated aurothiomalate to more effectively treat rheumatoid arthritis. Immunization of certain strains of mice and rats with heterologous type II collagen produces a form of rheumatoid arthritis that resembles the human form of the disease [6,11]. The immune response is T-cell dependent. $CD4^+$ lymphocytes and IL-2 receptor positive lymphocytes are soon detected after immunization [3]. Anti-collagen antibodies can be found in the sera of susceptible mice. By day 30 after antigen sensitization, TGF β-2, and the cell surface antigens Ia, Mac-1, and Gr-1 can be found in the synovium [22–25]. Several days later, TNF, IL-6, and $CD4^+$ cells are detected. These surface markers or cytokine-associated with T-cells tend to increase by day 40 and then level off while macrophage associated markers remains high.

Liposomes have successfully been used to encapsulate toxic drugs and deliver them as a non-toxic and more effective therapeutic [26–31]. As an example, the anti-fungal agent amphotericin B has been encapsulated into small unilamellar vesicles (SUVs). This complex is now used to treat deep tissue fungal infections with significantly less toxicity than the free drug. Liposomes with interleukin-2 coupled to the outer leaflet of SUVs bind to the high affinity form of the IL-2 receptor and can be used as a targeted form of a drug delivery vehicle [26]. Liposomes have been shown to traffic to the synovium of rodents and humans [32] Clodronate, an anti-arthritic, is being evaluated in a liposome-encapsulated form to suppress CIA in a rat model of arthritis [33]. The beneficial effects of this liposome-encapsulated drug complex to successfully reduce swelling of the joints may provide further insight into the cellular mechanisms of immune suppression and reduction of joint destruction.

In the past, the gold drugs have been evaluated in collagen-sensitized mice and rats with mixed results [34–36]. Here we present this study which shows that aurothiomalate encapsulated in SUVs effectively suppresses type II collagen-induced rheumatoid arthritis in DBA/1J male mice and also suppress other systemic effects of the disease. The successful outcome of the studies using a liposome-encapsulated form of ATM indicates that efficacy, as an anti-arthritic may best be achieved when the drug is shielded from the interstitial tissues prior to delivery to the synovium or to tissue phagocytes.

2. Materials and methods

2.1. Lipids

Hydrogenated egg phosphatidylcholine was purchased as a dry powder from Avanti Polar Lipids (Birmingham, AL). Cholesterol, 99%+ was from Sigma. All lipids were stored at −20°C. Gold sodium thiomalate was purchased from Pasadena Laboratories (San Clemente, CA) in vials of 50 mg/ml in water.

2.2. Mice

Male DBA/1J(H-2^q) mice were from Jackson Laboratories (Bar Harbor, ME) and were used at 6 months of age.

2.3. Preparation and characterization of liposome-encapsulated aurothiomalate: preparation of SUV ATM

Gold sodium aurothiomalate (500 mg) was lyophilized to dryness to remove butanol (0.5%) added by the manufacturer. Immediately before the preparation of liposomes, the drug was hydrated at 37°C in 1.0 ml 0.01 M HEPES (Sigma)–9% sucrose, pH 7.4 at 37°C. Small unilamellar liposomes were prepared using HEPC and cholesterol at a molar ratio of 2:1. 40 mg of total lipid were dissolved in 2 ml of chloroform/MeOH (2:1, v/v) and evaporated to dryness under nitrogen at ~42°C. The lipid film was then further dried under vacuum overnight to remove all traces of chloroform.

The lipid film was hydrated by incubation in 1 ml of HEPES-sucrose at 56°C for 10 min with occasional mixing. The solubilized ATM was then added and the mixture incubated for 5 min. Small unilamellar liposomes were made by sonication using a probe sonicator (50 mHz, Branson Sonifier) at room temperature for 10 min. Drug containing SUVs remained in the supernatant after centrifugation. The SUVs were further incubated at 56°C for 10 min, centrifuged at 14 000×g, 2 min. Non-encapsulated ATM was removed by chromatography over Sephadex G50-80 in the same buffer. Liposome-encapsulated ATM was stored under nitrogen at 4°C.

2.4. Encapsulated drug concentration

The concentration of ATM was determined by plasma emission spectroscopy (Quest Laboratories, Fort Collins, CO). The phospholipid concentration in SUV preparations was approximately 5 mg/ml total lipid.

2.5. Determination of vesicle stability

The integrity of the phospholipids monitored by thin layer chromatography. Aliquots of SUV ATM were applied to Silica Gel (Whatman) thin layer plates and chromatographed in $CHCl_3$/MeOH (7:3) along with purified lipid standards. The plates were developed by spraying with 50% (v/v) H_2SO_4 and heating. The retention of the encapsulated ATM was determined by filtration. The liposome-encapsulated drug was stored under nitrogen at 4°C. At various times after storage, aliquots of SUV ATM were diluted into HEPES-sucrose buffer and centrifuged at 2900 rpm in Centricon 30 filter units for 60 min, 4°C. The gold concentration of the filtrates was determined as described for SUVs and corrected for dilution.

2.6. Determination of vesicle diameter

Samples of SUV ATM were diluted with 2% phosphotungstic acid and examined by electron microscopy at 55 000× magnification. The diameter of the vesicles was measured using a scale of 18.1 nm/mm.

3. Induction of collagen-induced arthritis and treatment with liposomal ATM

Male DBA/1 mice were injected intradermally at the base of the tail with 100 μg of chick Type II collagen (Sigma)in 0.1 N HOAc. which had been emulsified in complete Freund's adjuvant (Sigma) All mice were boosted with 100 μg of collagen emulsified in incomplete Freund's adjuvant (Sigma) on day 21 after the initial sensitization. Mice were treated with SUV ATM or ATM in HEPES-sucrose by injection in one quadracep muscle (i.m.). Subsequent injections were given to alternating quadracep muscles. Treatments were initiated on day 1 at the same time as the initial antigen challenge. Mice were treated once per week thereafter. Mice were bled intermittently to assess anti-collagen antibody production. At the completion of the studies, animals were killed and splenocytes isolated and subcultured for subsequent in vitro studies.

3.1. Arthritic index

All groups of mice were observed weekly for signs of redness and swelling in the fore and hind paws.

The same individual always observed the mice while two other scientists not involved with the study [32,59–61] verified the symptoms of CIA. It was observed those collagen-sensitized animals would occasionally have red and swollen eyelids in addition to severely swollen feet. The criteria for ranking the development and extent of arthritis were as follows: each foot of an animal was either normal (score = 0), or red (+1). The degree of swelling was ranked as mild (+1), moderate (+2), or severe (+3). The development of arthritis followed a characteristic pattern of redness followed by progressive swelling, which was easily distinguished. Each foot had a maximal score of (+4). If an animal had an inflamed eye it received an additional (+1). The maximum score possible was 4×4 (feet)+2 (eyes) = 18. The arthritic index was = $(x/18)\times100$. The data from all treatment groups were analyzed using ANOVA and Tukey–Kramer HSD statistical methods to evaluate encapsulated drug reduction of collagen-induced arthritis.

3.2. Histological examination

At the end of a study, the hind legs and feet of mice from each treatment group were removed and quick-frozen in HistoPrep (Fischer Scientific). Samples were stored at −80°C until sectioning and staining in Hematoxylin–Eosin. Slides were examined and photographed at 40× magnification.

3.3. Anti-collagen EIA

Immulon EIA plates were coated with chick type II collagen (1 μg/ml) in 0.01 M sodium carbonate, pH 8.2, 4°C, overnight. Plates were washed with phosphate-buffered saline, pH 7.4–0.2% Tween 20, and blocked for 60 min with 3% ovalbumin/PBS. Plates were washed with PBS-Tween, and incubated with dilutions of antisera for 60–120 min, 37°C. Plates were again washed, and incubated with goat-anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotechnology) at a dilution of 1:300. Color development was initiated by the addition of *p*-nitrophenylphosphate (Sigma), 1 mg/ml in diethanolamine buffer, pH 8.6. Absorbance was read at 450 nm.

4. Results

4.1. Preparation and characterization of SUV ATM

Sodium aurothiomalate was easily encapsulated into SUVs and the protocol was extremely reproducible. These SUVs were quite stable during storage at 4°C with little leakage of aurothiomalate. This is shown in Fig. 1. Only 0.09% of the encapsulated drug leaked out during a 7-day period. Thin layer chromatography was used to monitor any gross changes in the oxidation state of the lipids. No major changes in the oxidation of hydrogenated egg phosphatidylcholine or cholesterol were found, although this is not a sensitive indicator of lipid oxidation. Preparations of SUV ATM were negatively stained with 2% phosphotungstic acid and observed by electron microscopy (Fig. 2). The average diameter was approximately 72.4 nm when SUVs were measured with this method (Fig. 2).

4.2. Development of CIA

The animal model of rheumatoid arthritis was established as described [20,21,36–38] and the observa-

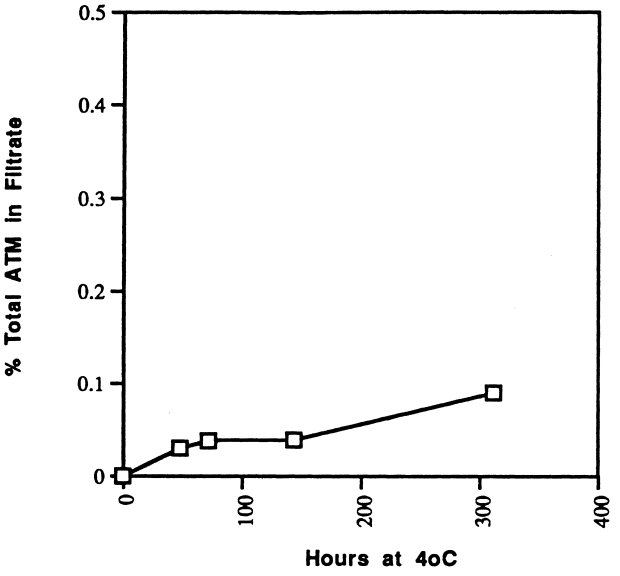

Fig. 1. Aliquots of SUV ATM were incubated at 4°C for the indicated intervals. A 50-μl amount of liposomes was added to 1.5 ml of HEPES-sucrose buffer and centrifuged at 2900 rpm, 4°C for 60 min. The filtrates from centrifugation were analyzed for the concentration of ATM as described.

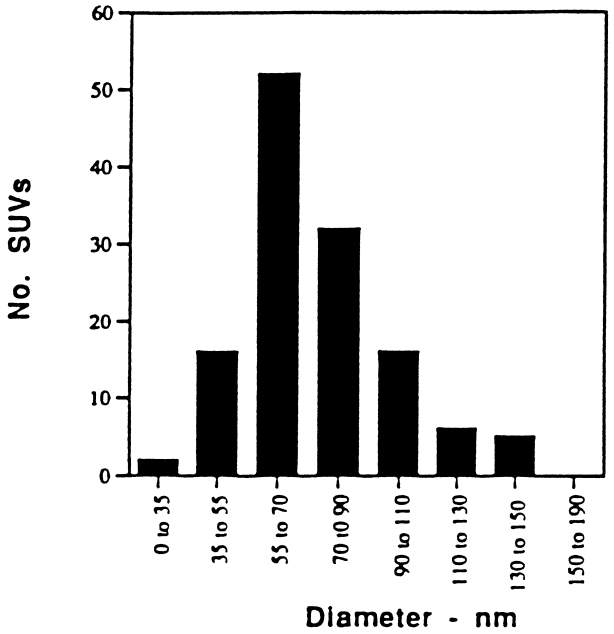

Fig. 2. A preparation of SUV ATM was stained with 2% phosphotungstic acid, and visualized by electron microscopy at 55 000×. The diameter of the vesicles was determined by comparison to the factor of 18.1 nm/mm.

tions of the symptoms were confirmed by three individuals. Redness of the fore paws and hind paws was detectable by day 8. All animals sensitized with chick collagen mounted an immune response and anti-collagen antibodies were detectable in the sera by day 32. The symptoms of collagen-induced arthritis consisted initially of red and then swollen hind feet by days 21–28. The disease then progressed to the front feet. Over time, the mice became less active. In addition, we observed that collagen-sensitized animals (without any drug treatment) occasionally had one or two red and swollen eyes, which varied in intensity and frequency of this reaction. All mice were boosted on day 21 with collagen and the studies were allowed to proceed for 70–80 days.

4.3. Treatment of collagen-sensitized mice with ATM or SUV ATM

The mice receiving either form of the drug were first injected on the same day that they first received collagen. This was done to see if ATM or SUV ATM inhibited the activation phase of antigen sensitization. Several pilot studies were carried out using small numbers of animals (five mice per treatment group) to determine if liposomal therapy would alter the development of arthritis. We observed that treatment with SUV ATM definitely suppressed the magnitude of arthritis in male DBA/1 mice. As compared to the collagen-treated mice (no drug treatment), the appearance of the hind feet as well as the fore paws of these mice receiving SUV ATM resumed a normal appearance and the foot pads were not swollen. Moreover, there was little residual redness. Two large-scale studies (12 mice per treatment group) were then carried out to follow this effect once it was seen that a distinct result was achieved with SUV ATM.

4.4. Escalating dose study

A range of liposome encapsulated drug concentrations was first chosen to determine how arthritic mice would respond to treatment and over what time period the symptoms of CIA would be altered. Fig. 3 presents the results of an 81-day study. The animals were both sensitized to collagen and treated on day 1. The concentration of drug per animal ranged from

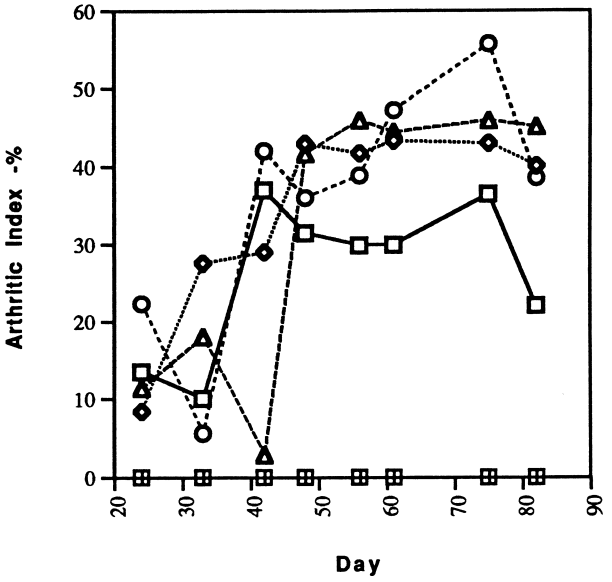

Fig. 3. The arthritic index from an escalating dose study of SUV ATM or ATM treatment of collagen-sensitized DBA/1 mice. Collagen (open circles), untreated mice (normals, (hatched squares), collagen+ATM treated (diamonds), collagen+ATM+SUV (triangles) and collagen+SUV ATM (open squares) present increased foot swelling during 81 days. The drug concentration ranged from 4 to 16 mg/kg per animal.

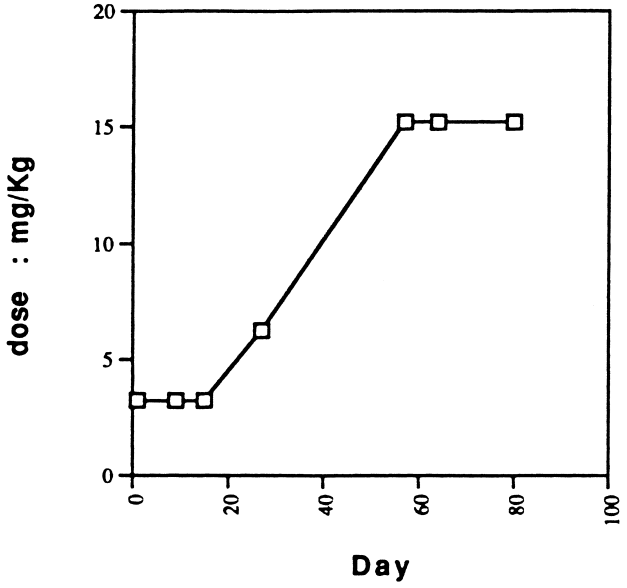

Fig. 4. The drug dose of SUV ATM or ATM during the study presented in Fig. 3 is shown in mg/kg of total body weight based on the weight of a 22-g mouse.

4 to 20 mg/kg (Fig. 4) over the duration of the experiment. There were five treatment groups consisting of untreated or normals, collagen-treated, collagen+ATM-treated, collagen+ATM+empty SUV-treated, and collagen+SUV ATM-treated. The last treatment group was included to compare the individual components of the encapsulated drug system.

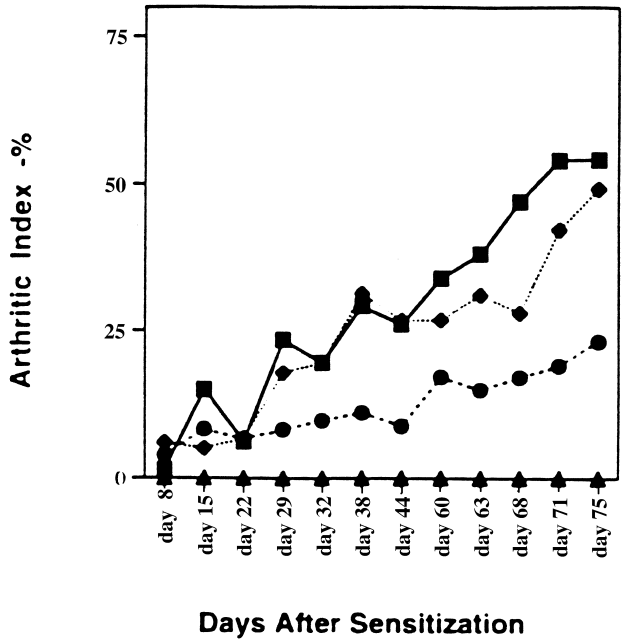

Fig. 5. The arthritic index of a elevated drug dose study. Collagen-sensitized mice were treated with 104 mg/kg for 81 days. Treatment groups were collagen-sensitized (filled squares), collagen+ATM (filled diamonds), collagen+SUV ATM (filled circles), and normals (filled triangles).

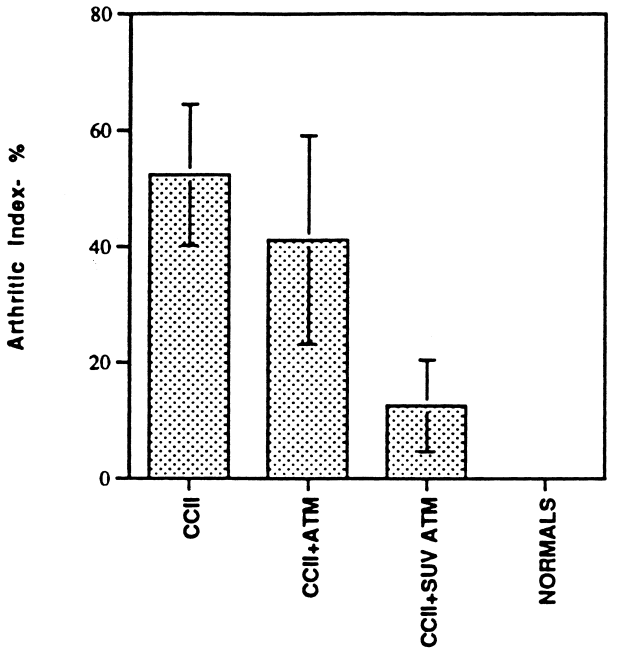

Fig. 6. A The comparison of symptoms among the treatment groups on day 81 from the study presented in Fig. 5.

As seen in Fig. 3, by day 75, the arthritic index of animals sensitized with collagen alone was almost 60% and all animals exhibited symptoms. Treatment of collagen-sensitized mice with ATM or empty SUV+ATM did not result in reduction of arthritic symptoms. Treatment of mice with collagen and SUV ATM reduced foot swelling. The arthritic index in SUV ATM-treated mice was 20% by day 75. The reduction in symptoms was evident by day 40 and remained at the same level for the duration of the study. Statistical analysis (ANOVA) of these studies on day 82 (Fig. 6) indicated that a comparison of collagen treatment versus collagen+SUV ATM demonstrated a P-value of 0.0004. Statistical analysis using the Tukey–Kramer HSD compares the difference of the means of each treatment group. The more positive the value, the more significant the comparison. Comparing the value between the SUV ATM treatment group to either the collagen or the collagen+ATM group gave a value 0.116516, which is significant. In contrast, comparing the collagen treatment group to collagen+ATM group gave a value of

0.066516. and a value of 0.024081 when compared to collagen+ATM+SUV group. Negative values arising from such a comparison are not significant. In this study group, treatment of collagen-sensitized mice with the 20 mg/kg of non-encapsulated drug (ATM) was without effect as any reduction of arthritic symptoms occurred.

4.5. Elevated dose study

This study consisted of four treatment groups: normals or untreated controls; collagen-treated; collagen+ATM-treated; and collagen+SUV ATM-treated. Animals were treated with 104 mg/kg of drug. As seen in Fig. 5, SUV ATM also reduced the magnitude of the arthritic index even when used at a 5-fold greater concentration than used in the previous study. The reduction in symptoms was already evident by day 29. The arthritic index of the mice treated with SUV ATM did increase somewhat during the course of the study, yet this group always exhibited far fewer symptoms over all. The data from day 80 (Fig. 6) were analyzed by ANOVA and the *P*-value comparing the statistical significance between the group treated with SUV ATM and the other treatment groups was <0.0001. There is little difference between the collagen treatment group and the collagen+ATM group. The Tukey–Kramer HSD value for significance was 0.247077 when the collagen group was compared to the collagen+SUV ATM group. When the collagen group was compared to the collagen+ATM group, a value of 0.102723 was obtained. The free drug suppressed symptoms only at the higher concentration, but was never as effective as the encapsulated drug in this regard.

None of the animals showed any overt signs of drug toxicity. All animals maintained the same weight from group to group. Those receiving SUVs had a slight weight gain that was not statistically significant. The mean weight of the mice at the end of the studies was 20–21 g. Reduction in activity was observed in all collagen-treated animals, but no loss of agility was observed in the arthritic mice. All mice were always able to readily retrieve food and water. Most importantly, the SUV ATM-treated mice were as active as control mice that had never been collagen-treated.

4.6. Histological examination of knee joints

The effect of drug therapy on cellular filtration into the joints of arthritic mice was assessed by microscopic examination of the knee joints. Mice from the dose escalation study where the drug dose varied from 4 to 20 mg/kg were killed on day 81. The hind legs and spleens were removed and the legs were quick-frozen and stored at −80°C. Examination of multiple slides from each of the treatment groups provided good documentation of the effect of drug therapy on cellular infiltration. Collagen-sensitized mice receiving no drug therapy had a significant degree of cellular infiltration into the synovium (Fig. 7A). A large zone of cells is present in the area below the synovial membrane (SM, Fig. 7A). Examination of the sectioned and stained joints revealed that collagen-sensitized mice treated with varying doses of SUV ATM (Fig. 7B) had minimal cellular infiltration. Samples from these mice were similar to the control or 'normal' mice, which had no collagen or drug therapy (Fig. 7C). The presence of osteoclasts can be seen in the bony end plate of the sample presented in Fig. 7C. The free drug (ATM) combined with empty SUVs was not therapeutic as demonstrated by the presence of cellular infiltration in the joint and no or little reduction in foot swelling (data not shown). It is clearly evident that those mice treated with SUV ATM had almost no lymphocytic infiltration in the synovium as compared to collagen-sensitized animals.

4.7. Immunological characterization of SUV ATM-treated mice

Obvious morphological changes in arthritic animals accompanied the treatment with SUV ATM and there was a noticeable reduction in both the swelling of the front and hind feet and inflammation of the eyes. Attempts were made to identify the target cell or biochemical pathway that was altered by SUV ATM. We chose to sample peripheral blood for anti-collagen antibodies. In addition, during the course of a study several animals of each group were killed on days 32 and 81 in order to isolate and sub-culture splenocytes in the presence of exogenous collagen to follow the production of cytokines

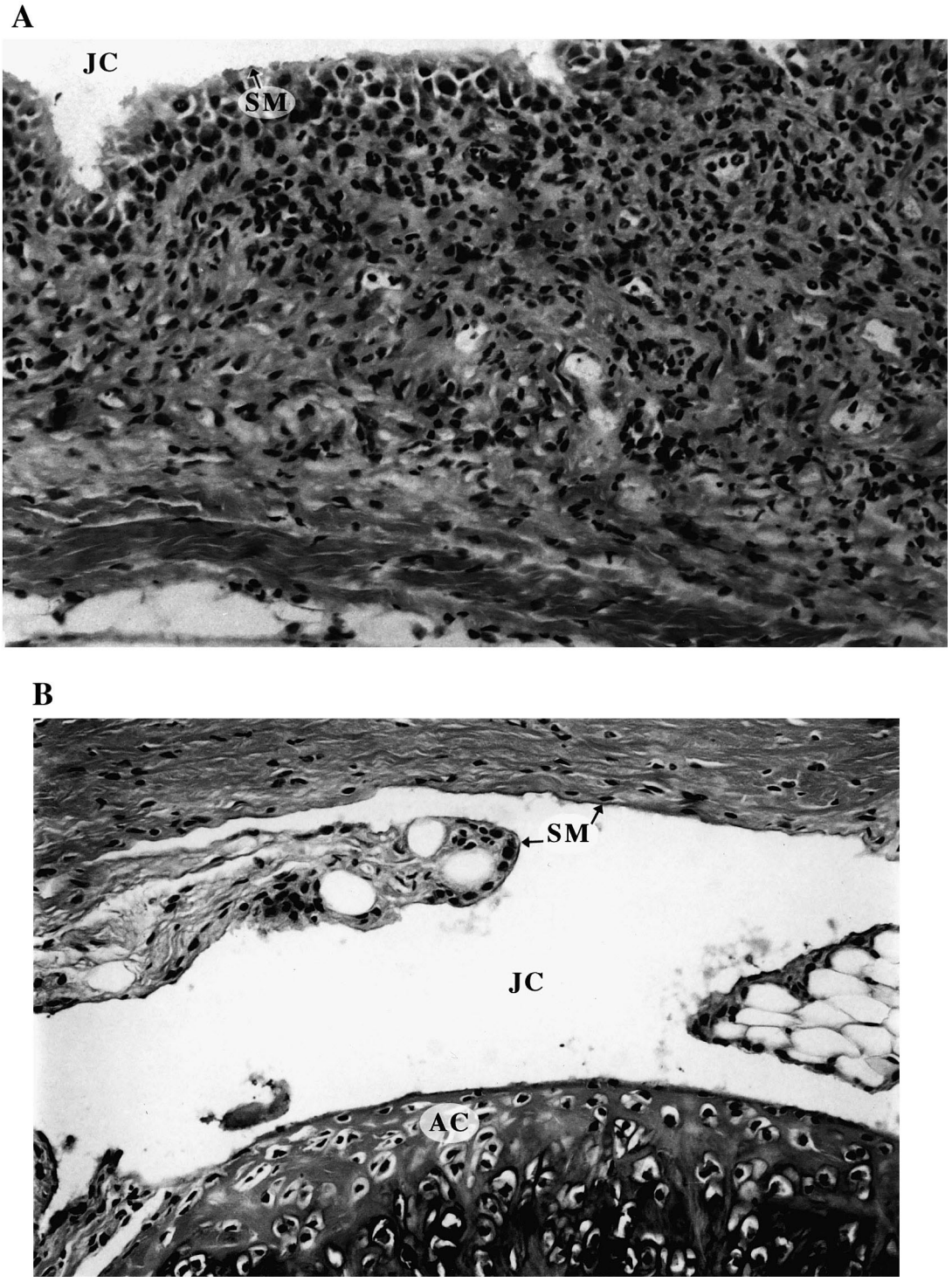

Fig. 7. (A–C) Histological sections of knee joints from collagen-treated and non-treated mice: (A) type II chick collagen treatment; (B) treatment with SUV ATM; and (C) normals or no collagen. Frozen knee joints were sectioned and stained with Hematoxylin–Eosin. JC is the synovial joint cavity, SM indicates the synovial membrane and AC locates the articular cartilage seen in C in non-collagen sensitized animals. 38×. Note the enhanced thickness of the synovial membrane in A.

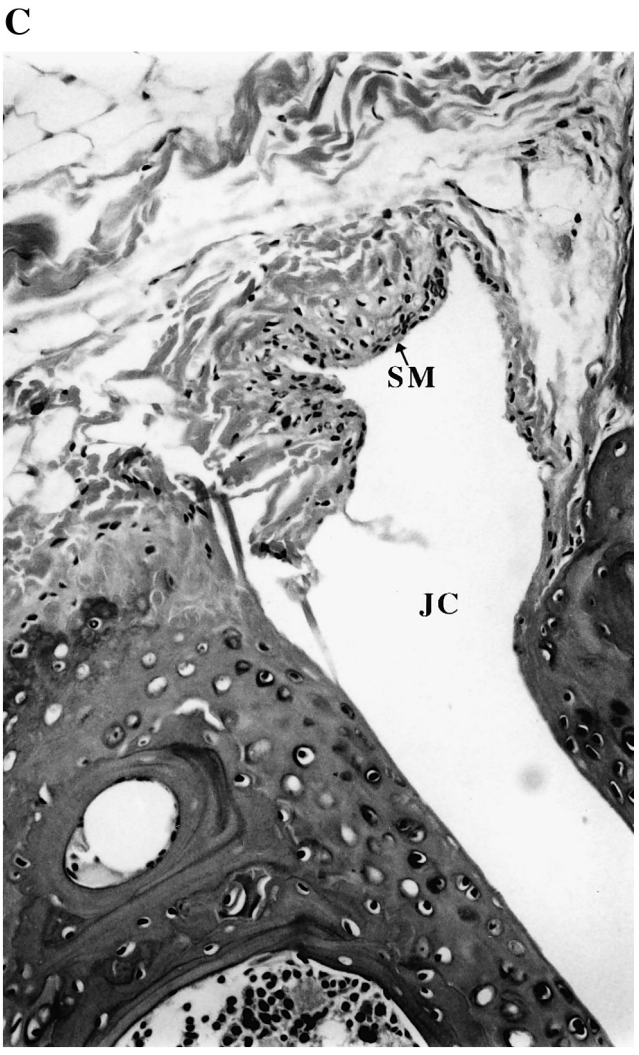

Fig. 7 (*continued*).

in the periphery in response to SUV ATM therapy in vivo.

The appearance of anti-chick collagen antibodies in the sera of the mice was followed by an ELISA for anti-collagen IgG. All mice sensitized to chick collagen had produced these antibodies. There was a very slight reduction of antibody production by drug therapy using SUV ATM (Fig. 8). The levels of anti-collagen antibodies remained elevated throughout the duration of the study in all groups of collagen-sensitized animals.

To assess lymphokine production in response to therapy, splenocytes were cultured in the presence of 10 μg/ml of chick collagen for 5–7 days. Aliquots of the conditioned medium from these cultures were assayed for the presence of IL-1β, IL-2, I-4, TNF, γ-IFN, TGF-β1, transferrin, and IL-10 using either bioassays (IL-2, TNF) or ELISAs (IL1-β, IL-4, γ-IFN, TGF-β1, transferrin, [41,42]. There was a slight reduction in the level of IL-4 when collagen-sensitized mice were treated with SUV ATM. There was no single cytokine in this group including IL-2 whose production was significantly affected by SUV ATM. All the other cytokines or acute phase proteins monitored were present and detectable, but were not significantly affected, positively, or negatively, by SUV ATM [19,24].

Lymphocytes were isolated from peripheral blood

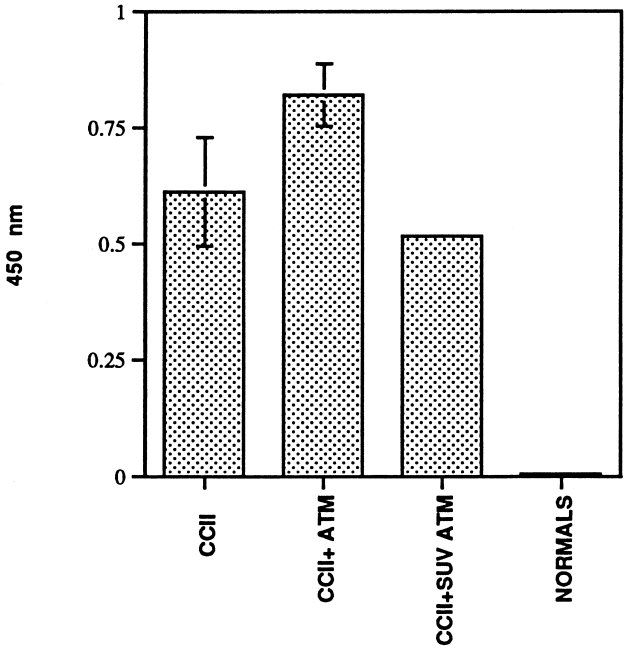

Fig. 8. The presence of anti-chick collagen antibodies in the sera of arthritic mice was detected by an ELISA using alkaline phosphatase-conjugated anti-mouse IgG1 antibodies (Southern Biotechnology). Mice were bled on day 32 after sensitization while being treated at an ATM dose of 104 mg/kg.

and stained for CD4, CD8 or the macrophage surface antigens F480 and MAC-1 to see if major changes could be detected in peripheral blood lymphocytes of treated versus non-treated CIA mice [3–6,22]. No major changes in these surface antigens were found. The ratio of $CD4^+$:$CD8^+$ cells was $\sim$2.5:1 consistent with the normal in vivo composition of these T-cell subsets. No altered distribution of macrophages in the peripheral blood was found evidenced by F480 or MAC-1 staining.

5. Discussion

Sodium aurothiomalate was easily encapsulated into stable small, unilamellar vesicles (SUVs) and this material was therapeutically effective. SUV ATM suppressed collagen-induced arthritis (CIA) in male DBA/1J mice (Figs. 3–5). The macroscopic signs of arthritis as well as cellular infiltration into the joints was significantly reduced by intramuscular injections of SUV ATM over an 81-day treatment period compared to non-treated animals having CIA. Suppression of arthritis was observed in both studies showing a broad range of the effective encapsulated drug dose. A 50% reduction in the symptoms was achieved at 16 mg/kg after 81 days. Increasing the injectable dose to 104 mg/kg did give a more defined pattern of suppression, but the higher dose produced a slight suppression in the presence of the free drug that was not observed in the first study (Figs. 3 and 5). The free-drug was only slightly effective in suppressing CIA at the higher concentration of 104 mg/kg. It is clear in this liposomal system that greater efficacy occurs using liposome-encapsulated drug than would be the case using ATM alone. This is a hallmark of successfully encapsulated drugs.

We observed a reduction in the local concentration of infiltrating lymphocytes present in the joint of the hind limbs when mice are treated with SUV ATM, but not with the free drug (Fig. 7A–C). The appearance of tissue samples from SUV ATM-treated mice was very similar to those of mice that had not received collagen injections. Moreover, the absence of cellular infiltration is directly correlated with a decrease in foot swelling and in a few of the animals there was a reduction in eye swelling. It has been observed that a reduction of macrophages in the perivascular space of the synovium is observed in samples of needle biopsies from ATM-treated patients who have had active rheumatoid arthritis [40].

We examined the capacity of lymphoid organs in the periphery modulate changes in the ability of resident T- and B-cells to produce inflammatory lymphokines. It was of interest to see if collagen-sensitized mice treated with SUV ATM would produce less IL-1β or TNF-α among others. We could not detect the presence of inflammatory lymphokines at concentrations above background. We assayed both sera and conditioned medium for TNF, transferrin, TGF-β1, IL-1, IL-2, IL-4 and IL-10.

An obvious cellular target for SUV ATM would be tissue and synovial macrophages [43–48]. Phagocytosis of SUV ATM would then lead to an alteration in the inflammatory cytokine profiles. Moreover, intact SUV ATM carried by the circulation can easily reach the synovium [32]. The average diameter of SUV ATM is $\sim$72 nm. It has been reported that liposomes 80–90 nm in diameter injected subcutaneously could be found in the blood 12–24 h postinjection [42]. Although SUV ATM is injected intra-

muscularly, it probably does not remain at the site of injection and in time, some could enter the synovium [32]. Biodistribution studies were not carried out to determine the concentration of the drug in the major organs and tissues among the treatment groups.

Within the synovium of humans with rheumatoid arthritis or mice with CIA there is a significant infiltration of lymphocytes (Figs. 3–5). The presence of both $CD4^+$ and $CD8^+$ T-cells, macrophages and B-cells in the synovium has been well-documented [39,40]. By day 30 after antigen sensitization, the lymphokine TGFβ-2 and surface molecules Ia^q, Mac-1, and Gr-1 can be found on cells that have infiltrated the synovium. Several days later, the lymphokines TNF, IL-6, and $CD4^+$ cells are detected. These surface markers or cytokine-associated with T-cells tend to increase by day 40 and then level off while macrophage associated markers Mac-1, Gr-1 remain high [39–41]. The identity of the autoantigen, which elicits the cycle of inflammatory processes in rheumatoid arthritis in humans, is unknown. Immunoreactive cells are present and active in the synovium even if the antigen is a self-antigen and elicits a MHC class I response [48] or whether a foreign antigen, such as collagen, *Mycobacteria*, or *Borellia*, is present leading to a MHC class II response.

At the present time, the understanding of ATM efficacy or toxicity is hindered by the identification of a defined biochemical or cellular target affected by the drug. Several cell types and many different biochemical systems appear to be altered by the gold drugs. The various systems affected by gold drugs include complement activation [49], collagenase secretion [50–52], macrophage and neutrophil activation [18,49,53], and inhibition of neutrophil chemotaxis [54], inhibition of lymphocyte proliferation and immunoglobin synthesis [54–58].

Interestingly, the gold drugs aurothiomalate and auranofin do not suppress collagen-induced arthritis in rodents in a consistent manner. Yet, use of liposome-encapsulated sodium aurothiomalate consistently reduced the symptoms associated with collagen-induced arthritis. Some investigators report that ATM does not suppress collagen-induced arthritis [59,60]. Others report that only auranofin exerts immunosuppressive effects in a rat model and does not work in a mouse model [61] Aurothiomalate seems to cause some toxicity among strains of mice [59–61]. Auranofin appears somewhat ineffective in reducing symptoms of arthritis in collagen-treated rats [59–61]. Aurothiomalate can suppress inflammatory responses elicited by carragenen in the footpad or the cheek pouch of the mouse [62]. A long-term treatment of three mouse strains with gold drugs was carried out to follow changes in pharmacokinetic profiles of the drug even though the mice are not sensitive to the drug [62].

Oddly enough, using murine lymphocytes, both aurothiomalate and auranofin can be studied in vitro and affect a variety of biochemical and immunological parameters. Suppression of lymphocyte proliferation, protein kinase C activity, glucocortico receptor activity and NF κB activity occur [18]. Again, these studies were performed using non-encapsulated drugs where charge effects may play a role in intracellular entry and a rodent's ability to respond to the drug.

It should be pointed out that in this liposomal system of drug delivery, efficacy was achieved at encapsulated drug concentrations where the free drug was ineffective.

We did not observe any overt signs of drug toxicity during the course of the work. All study groups including the negative control group maintained their weight ($\sim$20–21 g). The collagen-treated mice exhibited a reduced level of activity relative to the negative controls as their feet became progressively swollen, but no other changes were observed. Collagen-treated mice that received SUV ATM were as active as non-collagen-treated ones. Irrespective of the specified cellular or biochemical target, encapsulation of ATM within the internal aqueous space of small unilamellar vesicles strongly enhances its efficacy in reducing the overt symptoms of type II collagen-induced arthritis reduced, shown by reduced foot swelling and a virtually no cellular infiltrate in the synovium. The study also suggests that the variability in the sensitivity of rodents to a drug such as ATM may reflect the fact that this derivative of malic acid does not easily cross the cell membrane. Obviously, encapsulation shields the cell from a charged molecule. Sodium aurothiomalate, auranofin, and sulfasalazine are part of a small number of anti-arthritic drugs having the capacity to retard the progression of rheumatoid arthritis at the cost of frequent drug toxicity. This work has shown that efficacy may be reduced by delivery into the cell when encapsulated.

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