

Evaluation of Cetyl Myristoleate in a Mouse Model of Rheumatoid Arthritis

FINAL REPORT

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2. Report Summary

This 6 month research project was designed to investigate the potential anti-arthritic effect of the carboxylic acid ester cetyl myristoleate (CM). The project had three major goals: organic

synthesis of CM and the related cetyl myristate; development of a gas chromatographic/flame ionization detection (GC/FID) technique for analysis of CM; and testing of CM in a mouse model of collagen-induced arthritis. The major findings of this study are summarized below.

- Reliable synthetic organic chemical methods for CM and cetyl myristate were developed. The purity of the resulting compounds were determined by thin layer chromatography, infrared spectroscopy, and both ^{13}C and ^1H nuclear magnetic resonance spectrometry. The synthetic method for CM, while yielding a pure product, requires relatively expensive starting materials and probably could not be adapted for large-scale production.
- A Gas Chromatography/Flame Ionization Detection (GC/FID) method was developed for the analysis of CM in human serum.
- Three experiments using mouse models of rheumatoid arthritis were performed.

1) In the first pilot study, monoclonal antibodies against bovine type II collagen (that cross-react with mouse type II collagen) were injected along with lipopolysaccharide into BALB/c mice. Two of the six mice in this pilot experiment were injected concomitantly with 450 mg/kg of CM via the intraperitoneal (i.p.) route. This method of inducing arthritis by injecting pre-formed antibodies caused fulminate arthritis within 5 days, and although there was no apparent effect of the CM treatment, the model was much too rapid and rigorous to have expected any palliative effect of the compound. However, the experiment did allow the development of an operant training regimen to assess the change in mobility of the mice as they developed painful inflammatory joint disease.

2) In the second study, arthritis was induced in susceptible DBA1/LacJ mice by the intradermal injection of bovine type II collagen emulsified in adjuvant. This collagen-induced arthritis model developed much more slowly, with the peak of disease around 6 weeks post-immunization. Beginning on day 12 post-immunization and continuing every third day until day 30, groups of mice were given i.p. doses of CM at either 450 mg/kg or 900 mg/kg. These dosages were in keeping with the dosages used in the original Diehl and May (1994) study that had ascribed an anti-arthritic effect to CM. It was discovered that when compared with phosphate buffered saline-treated control mice, both the 450 and 900 mg/kg doses of CM reduced the incidence of arthritis, and also reduced the severity of the arthritis in those mice that showed signs of this inflammatory disease.

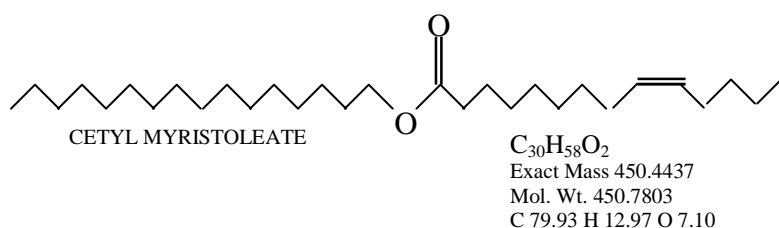
3) A third study was done using the collagen-induced arthritis model. However, in this study, the mice were also trained to perform a characteristic movement in an automated, video-monitored operant chamber in order to determine whether CM could positively affect the pain associated with the arthritic condition. DBA1/LacJ mice were given CM orally to humans in daily doses consistent with the amount of CM given orally in the commercially available product MysterolTM. The results from this study indicated that oral CM reduced the incidence and magnitude of arthritis in the mice, albeit less significantly than the reductions seen with the higher doses of CM given via the i.p. route in the first collagen-induced arthritis study. The movement data correlated with the development of clinical signs and also depicted the apparent benefit of CM treatment.

3. Project I – Synthetic Chemistry

The first task in the project was to synthesize approximately 10 grams of pure cetyl myristoleate, the active ingredient of the Sierra Life Sciences product Mysterol™. In addition, 10 grams of cetyl myristate, a related compound that is also a substantial component of Mysterol™, was synthesized. The synthesis of both compounds was performed in the laboratory of Dr. Suk-Wah Tam Chang, Associate Professor of Chemistry at the University of Nevada.

3.1 Synthesis and Characterization of Cetyl Myristoleate

The structure of cetyl myristoleate is depicted below:



3.1.1 Analysis of the Synthetic Product

A variety of standard analytical techniques were used to characterize the synthetic product. The results of these analyses are shown below

Nuclear Magnetic Resonance (NMR) Spectroscopy:

¹H NMR_ Instrument: Varian Unity Plus

¹H NMR (500 MHz, CDCl₃) 5.36-5.33 (m, 2 H, *J* = 3 Hz), 4.05 (t, 2 H, *J* = 6.8 Hz), 2.29 (t, 2 H, *J* = 7.8 Hz), 2.01 (m, 4 H, *J* = 3 Hz), 1.61 (m, 4 H, *J* = 6.8 Hz), 1.30-1.26 (br d, 38 H), 0.89 (q, 6 H, *J* = 7.5 Hz).

This data indicated that the protons of the CH₂ group adjacent to the ester oxygen are split into a triplet and resonate at 4.05 ppm. This is consistent with known literature values for **esters**.

¹³C NMR Instrument: Varian Unity Plus

¹³C NMR (125 MHz, CDCl₃) 174.16, 130.13, 129.97, 64.61, 34.62 32.18, 32.15, 29.92, 29.91, 29.90, 29.88, 29.87, 29.80, 29.75, 29.58, 29.48, 29.38, 29.35, 29.32, 28.88, 27.37, 27.13, 26.16, 25.23, 22.91, 22.56, 14.32, 14.20.

This data indicated that the carbonyl carbon of synthetic product resonated at 174.11 ppm, and this is consistent with the known literature values for ester carbonyl carbon resonances. In addition, the resonance at 64.61 ppm is consistent with known literature values for the CH₂ carbon adjacent to the ester oxygen.

Infrared Spectroscopy (IR)

Instrument: Perkin Elmer Spectrum 2000 FT-IR

IR (Neat/NaCl) 2924 (alkane C-H stretch), 1740 (ester C=O stretch), 1655 (disubstituted cis C=C stretch), 721 (disubstituted cis alkene C-H out-of-plane bend) cm^{-1} .

Instrument: Perkin Elmer Spectrum BX FT-IR

IR (KBr/ CH_2Cl_2) 2928 (alkane C-H stretch), 1739 (ester C=O stretch), 1654 (disubstituted cis C=C stretch), 722 (disubstituted cis alkene C-H out-of-plane bend) cm^{-1} .

The absorption at 1740 cm^{-1} is consistent with known literature values for an ester C=O stretch. In addition, the absorptions at 1654 and 722 cm^{-1} are consistent with known literature values for a cis disubstituted alkene C=C stretch and C-H out-of-plane bend, respectively.

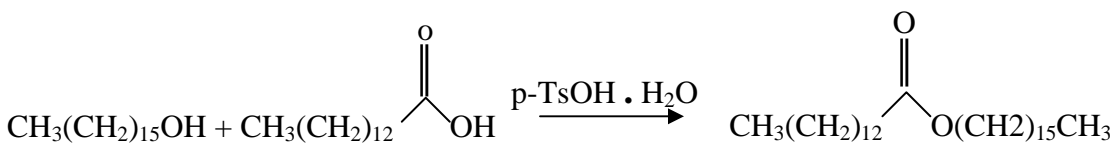
Thin Layer Chromatography (TLC)

TLC Plate: EMI Science Silica Gel 60 F₂₅₄ 250 μm thick

R_f = 0.58 (CHCl_3) Visualization: Cerium sulfate/Ammonium molybdate in conc. H_2SO_4 .

Conclusion. The results of these analyses indicate that the synthetic procedure described yielded a product consistent with the known structure of cetyl myristoleate.

3.2 Synthesis of and Characterization of Cetyl Myristate



The synthetic scheme for cetyl myristate is shown below:

Myristic acid (10.46 g, 45.8 mmol), cetyl alcohol (15.61 g, 64.4 mmol, 1.4 equiv.), and p-toluene sulfonic acid monohydrate (6.95 g, 36.5 mmol, 0.8 equiv.) were dissolved in 700 ml benzene and heated at reflux under $\text{N}_2(\text{g})$ for 22 h with azeotropic removal of water. The solvent was removed to give an off-white solid that was dried under vacuum for 26 h. The solid was dissolved in 100 ml CHCl_3 and vacuum flash chromatographed on silica (11 x 11 cm, 32-63 μm particle size, 60 \AA pore size). The solvent was removed to give a clear oil that solidified upon cooling. The solid was dried under vacuum for 19 h to afford 17.75 g (39.2 mmol, 86%) of a white solid.

3. Analysis of the Synthetic Product

The same analytical techniques used for cetyl myristoleate were used for cetyl myristate.

Nuclear Magnetic Resonance Spectroscopy (NMR):

^1H NMR Instrument: Varian Unity Plus

^1H NMR (500 MHz, CDCl_3) 4.05 (t, 2H, $J = 7$ Hz), 2.29 (t, 2H, $J = 7.5$ Hz), 1.61 (m, 4 H), 1.26 (m, 46 H), 0.88 (t, 6 H, $J = 7$ Hz).

The protons of the CH_2 group adjacent to the ester oxygen are split into a triplet and resonate at 4.05 ppm that is consistent with known literature values for esters.

^{13}C NMR Instrument: Varian Unity Plus

^{13}C NMR (125 MHz, CDCl_3) ? 174.22, 64.61, 34.65, 32.16, 29.93, 29.91, 29.88, 29.84, 29.81, 29.76, 29.71, 29.59, 29.51, 29.49, 29.40, 28.89, 26.17, 25.27, 22.92, 14.33.

The carbonyl carbon of cetyl myristate resonates 174.22 ppm that is consistent with known literature values for ester carbonyl carbon resonances. In addition, the resonance at 64.62 ppm is consistent with known literature values for a CH_2 carbon adjacent to an ester oxygen.

Infrared Spectroscopy:

Instrument: Perkin Elmer Spectrum BX FT-IR

IR ($\text{KBr}/\text{CH}_2\text{Cl}_2$) 2916, 2848 (alkane C-H stretches) 1732 (ester C=O stretch) cm^{-1} .

The absorption at 1732 cm^{-1} is consistent with known literature values for an ester C=O stretch.

Thin Layer Chromatography (TLC):

TLC Plate: EMI Science Silica Gel 60 F₂₅₄ 250 mm thick

$R_f = 0.6$ (CHCl_3) Visualization: Cerium sulfate/Ammonium molybdate in conc. H_2SO_4

The results of these analyses indicate that the synthetic procedure described yielded a product consistent with the known structure of cetyl myristate.

4. Project II – Analytical Chemistry

4.1 Development and Testing of a Gas Chromatographic/Flame Ionization Detection Method for Cetyl Myristoleate

To facilitate future studies of the pharmacokinetics and pharmacodynamics of CM in humans, a sensitive analytical method is needed. The laboratory of Dr. Glenn Miller was tasked with the development of such a method for CM, including the procedure for extracting CM from serum.

4.1.1 GC/FID Method Development

The first experiment involved the analysis of pure CM in an HP5890 Series II Gas Chromatograph with Flame Ionization Detector. The detector utilized a 0.53 mm i.d. X 10 m HP1 column. The starting temperature was 200°C, and was programmed to increase at 10°C per minute to 300°C. For this pilot experiment, a 5 µl volume of CM in pentane (ca. 10 ppm) was injected into the GC. It can be seen from the profile shown in Figure 4.1.1., CM elutes at approximately 9.3 minute as a distinct peak on the chromatogram. This method is clearly capable of identifying this fatty acid ester.

4.1.2 Analysis of Cetyl Myristoleate in Human Serum

Under Patent Filing

4. Project III – Testing of Cetyl Myristoleate in a Mouse Model of Rheumatoid Arthritis

The mouse serves as a good model of rheumatoid arthritis in humans (Trentham et al., 1977; Courtenay et al., 1980; Wooley, 1988). Literally hundreds of scientific papers have been published using mice, with arthritis induced in several ways. For our research, we have employed two methods for inducing arthritis; immunization with pre-formed antibodies to type II collagen, and immunization with type II collagen in adjuvant. In both cases, the anti-collagen antibodies bind to type II collagen in the synovia of the joints, and such binding precipitates a local inflammatory response characterized by macrophage activation, release of proinflammatory cytokines, neutrophil and macrophage-mediated destruction of the synovia, and eventually erosion of the cartilage and bone. Obviously, the time course of inflammation is more rapid following the injection of pre-formed antibodies, but the pathology seen in both schedules of immunization is similar.

A. Pilot Project Using the Chemicon Arthrogen-CIA® Arthritis-Inducing Monoclonal Antibody Cocktail in BALB/c Mice

An initial study was performed to evaluate a computerized operant training system for assessing arthritis-induced movement impairment, to perfect the methods for clinical evaluation of arthritis, and to work out the procedures for joint histopathology.

5.1.1 Study Design and Procedure of Inducing Arthritis

The design of this experiment is shown in Table 5.1.

Table 5.1. Study Design for the Pilot Monoclonal-Induced Arthritis Project

Mouse ^a Number	Operant Training Regimen ^b	Treatment ^c
1	Lever Press	450 mg/kg CM i.p.
2	Lever Press	normal saline i.p.
3	Lever Press	normal saline i.p.
4	Topography	450 mg/kg CM i.p.
5	Topography	normal saline i.p.
6	Topography	normal saline i.p.

- a) Eight week-old female BALB/c mice were used in this experiment (see text for description of the monoclonal antibody-induction of arthritis)
- b) Two different operant training regimens were employed (see text for description)
- c) The drug was delivered in a volume of 100 μ l

To induce arthritis rapidly for this pilot study, we used the Chemicon Arthrogen-CIA® Arthritis-Inducing Monoclonal Antibody Cocktail in BALB/c mice (Terato, et al., 1992; Terato et al., 1995; Terato et al., 1996). In this procedure, a cocktail of mouse monoclonal antibodies raised against various components of bovine type II collagen was injected into recipient mice (2 mg/mouse in PBS given i.v.). These preformed antibodies recognized and bound to type II collagen in the joint synovia of the mice, rapidly causing an inflammatory synovitis. The inflammation was enhanced by injection of bacterial lipopolysaccharide (endotoxin, *Escherichia coli* O11:B4, 50 μ g in 0.1 ml PBS given i.p.) on days two and three. The progression of the inflammatory joint disease was very rapid with signs of swelling and erythema visible in four or five days (refer to section 5.2.2. for description of clinical scoring)



Fig 5.1.2.1. Close-Up of Chamber

5.1.2 Operant Training of BALB/c Mice

Animal models of arthritis routinely assess the progression of disease by observing and/or measuring the inflammatory changes in affected joints. This can be done by clinical observations, sometimes supplemented with direct measurements of joint swelling. However, it is known that pathological changes preceding the overt changes in joints occur both in humans and animals with arthritis. However unlike in human patients, it is not possible to ask the subject mice how their joints are feeling, so changes in

behavior often substitute as an assessment of pain. While some authors have used direct observations of the behavior of the animals in an attempt to assess pain, these methods are subjective and have not provided consistent results. We have developed a video-monitored, computer-controlled operant conditioning chamber that can automatically shape the behavior of mice and assess changes in behavior over time without human observational bias (see Figures 5.1.2.1. and 5.1.2.2.). Our goal in this pilot experiment was to determine whether changes in conditioned movement behavior could be used to assess the early onset in inflammatory joint disease.

Two protocols were employed for conditioning the movement behavior of the subject mice. First, a protocol used in which the mice were trained to press a lever at the of the operant chamber in order to produce a food pellet reward at the front of the chamber. Upon successful press, a tone would sound and the animal would then to the food hopper or cup to receive a food pellet. A very regularized movement between the lever and the food hopper was seen, and this movement was altered by the arthritis (see below).



was
back

lever
move

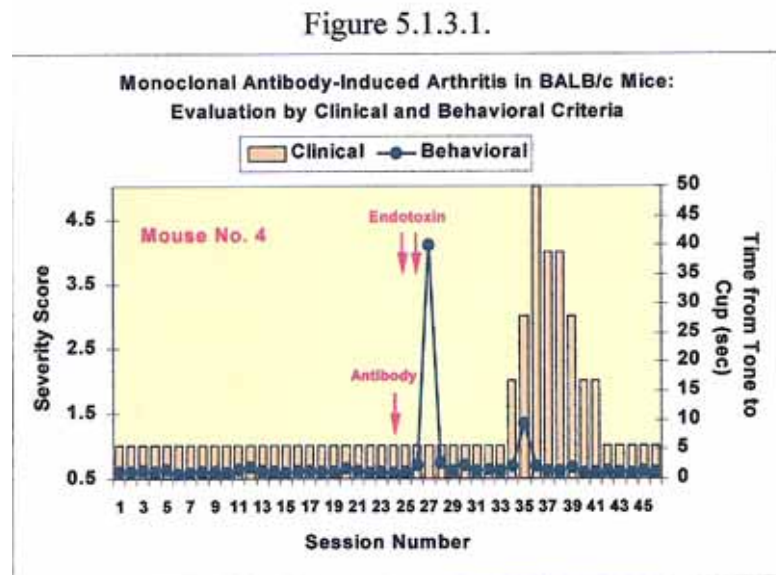
Fig. 5.1.2.1. Operant Conditioning Chamber

In the second protocol, the behavior of the mice was gradually shaped to a desired topography consisting of a left hand turn from the food hopper, movement down the left side of the chamber, and another left turn to the back of the chamber. In both protocols, the mice were food deprived overnight to encourage performance in the operant chamber. After each days' session, the animals were fed *ad libitum* from 1 pm to 7 pm. Under this regimen of food deprivation, the animals gained weight at approximately 90% of their food non-deprived littermates. Note that this is an acceptable weight difference in such animal behavior studies

5.1.3 Clinical/Behavioral Outcome and Effect of Cetyl Myristoleate

Injection of pre-formed anti-collagen antibody followed by endotoxin created a very rapid and progressive inflammation in five to seven days (see Figures. 5.1.3.1 and 5.1.3.2). In this pilot experiment, the behavior of the mice had attained a steady state over the first 24 sessions (note that each session is a 21 minute training period on one day).

The time from tone to arrival at the cup was measured and it can be seen in the Figure that the endotoxin injection caused a profound increase in the interval. However, the mice rapidly recovered from early effects of the endotoxin treatment (mild septic shock), and for



several days their behavior returned to steady-state level. When the acute joint inflammation induced by the antibody and endotoxin treatment began, the mice once again demonstrated a prolonged interval from tone to cup, indicating that this system can identify mice that are suffering from joint disease. Although the operant conditioning protocol allowed us to see the effects of this radical disease treatment slightly before it was apparent from clinical observations, the progression was far too fast to make meaningful sense of this data. In addition, there was no effect of the 450 mg/kg CM in either of the two treated mice. However, the fulminate nature of this disease model probably would have overshadowed the effect of any anti-inflammatory treatment. Nevertheless, this experiment allowed us to evaluate the operant conditioning model, working out the details of data collection and analysis.

5.2 First Collagen-Induced Arthritis Study in DBA1/LacJ Mice

This model was first described by Courtenay et al. (1980) with subsequent work on genetics and immune responses by several other groups (Wooley et al., 1981; Terato et al., 1985; Brand et al., 1994). The susceptibility of mice to collagen-induced arthritis is strongly linked to the major

histocompatibility complex (MHC), with mice of the H-2^q and H-2^f haplotypes being most susceptible. Perhaps the most commonly used mouse strain for these studies is the DBA/1LacJ mouse, and it is this mouse strain that I have chosen for these experiments.

5.2.1 Study Design and Procedure for Inducing Arthritis

Table 5.2.1 Study Design

Group	Mice	Treatment	Treatment Days
1	10	PBS Control i.p.	12, 15, 18, 21, 24, 27, 30
2	10	450 mg/kg CM i.p.	12, 15, 18, 21, 24, 27, 30
3	10	900 mg/kg CM i.p.	12, 15, 18, 21, 24, 27, 30

Thirty male DBA/1LacJ mice were injected intradermally at two sites on their shaved backs with a total of 100 mg of bovine type II collagen emulsified in complete Freund’s adjuvant (CFA). This treatment caused the mice to produce antibodies that interacted with their own synovial collagen, precipitating an inflammation of the joints (rheumatoid arthritis). The mice were randomized into three groups of 10 mice, and on day 12 each group was injected via the intraperitoneal route with 100 µl of either phosphate buffered saline (PBS, vehicle control), 450 mg/kg cetyl CM, or 900 mg/kg CM. The PBS or CM injections were repeated every third day until day 30 post arthritis induction (7 total doses). Note that the dosages of CM were chosen based on dosages used in the original Diehl study (Diehl and May, 1980). It should also be noted that CM is very insoluble in PBS, so the correct dose was added as a layer on 100 µl of PBS, pulled into a 1 cc syringe, and injected as a “suspension” of CM in PBS.

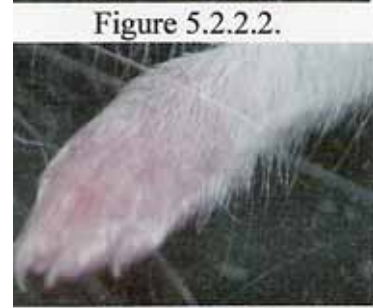
5.2.2 Clinical Evaluation of Arthritis

The mice were observed daily for signs of arthritis (note that these animals had not undergone operant training for movement). The standard 5-point clinical scale for assessing the degree of arthritis was used (see Table 5.2.2):

Table 5.2.2. Clinical Scale for Assessing Development of Arthritis in Mice

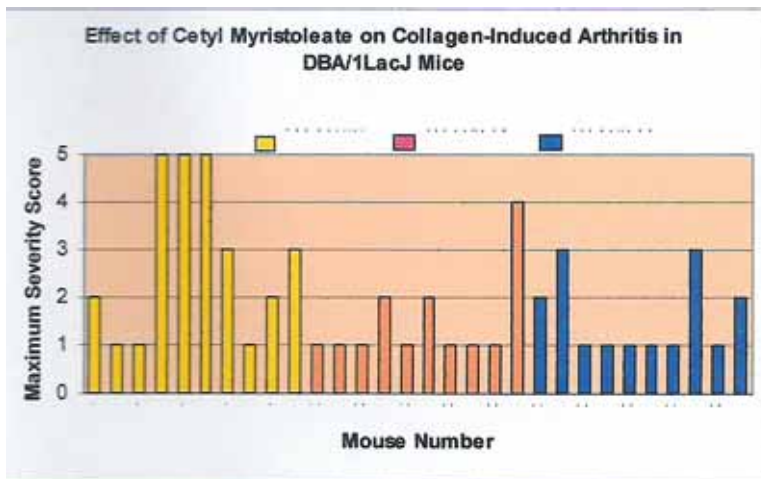
1	No evidence of erythema or edema
2	Erythema and mild edema confined to midfoot (tarsals) or ankle joint, or front paw and wrist (carpals)
3	Erythema and edema extending from the ankle joint to the tarsals, or from the elbow joint to the carpals
4	Erythema and moderate edema from the ankle to the metatarsal joints, or from the elbow to the metacarpal joints
5	Significant erythema and edema of all major joints of at least one limb

While the data can be portrayed in many ways, I have chosen to use the **maximum severity score**. This indicator does not assess the rate at which arthritis develops, but rather looks at maximum severity of the clinical disease at any time in the course of the experiment. The earliest sign of arthritis in the mouse model is demonstrated in Fig. 5.2.2.1. The slight swelling (edema) and redness (erythema) can be seen in the digits of the front paw. This effect is most noticeable in white mice (BALB/c), and more difficult to discern in brown grey mice (DBA1/LacJ). Note that there is no swelling between the wrist joint and the elbow joint in this example. In contrast, a much more severe swelling can be seen in Fig. 5.2.2.2. In this mouse, the digits of the front paw are grossly edematous, and the swelling extends from the wrist to the elbow. Indeed, the elbow joint in this animal is frankly one limb is affected. However, in the case that more than one limb is swollen or reddened, they usually ipsilateral.



Further progression of the inflammatory process leads to the clinical presentation shown in Figure 5.2.2.3. Here, the inflammation extends from the digits to the ankle, and the erythema is bright red. Though it is not apparent in this photo, the swelling extends to the knee joint. Mice with this degree of inflammation have very definite impairment in mobility, and may also exhibit systemic effects of the arthritis (e.g., lethargy).

5.2.3 Clinical Outcome and Effect of Cetyl Myristoleate



The data from this second arthritis experiment is shown in the Figure 5.2.3 below. Seven of 10 control animals (70%) showed signs of arthritis, which is consistent with published results using this collagen-induced arthritis model. In the 450 mg/kg and 900 mg/kg CM-treated groups respectively, only 3 of 10 and 4 of 10 mice developed signs of arthritis. The most severe arthritis was evident only in the PBS control mice

(maximum severity score of 5 in three mice). The average (\pm SD) severity scores for the PBS control, 450 mg/kg CM-treated, and 900 mg/kg CM-treated mice were 2.8 ± 1.6 , 1.5 ± 0.92 , and

1.6 ± 0.84 respectively. Using a paired, two-tailed t test, the probability of a difference in the means was p = 0.03 for PBS vs. 450 mg/kg CM, and p = 0.13 for PBS vs. 900 mg/kg CM.

5.2.4 Histopathology

To verify that our mouse models of arthritis resulted in pathologic changes similar to RA in humans, we performed necropsies on normal and arthritic mice. Gross examination of affected limbs revealed significant erythema and edema characteristic of inflammatory joint disease. Ankle joints were processed for histopathologic examination using the following method. Donor mice were sacrificed with halothane, and then a portion of their hind limb extending 2 mm proximal and distal to the ankle joint was excised and placed in 10% neutral buffered formalin. After fixation, the bone was decalcified using Decal-Stat, then the tissues were processed in an automated tissue processor with final embedding in paraffin. Sections were cut with a microtome at 7.5 µm, mounted on slides, then stained with standard haematoxylin and eosin (H&E). Thin sections were examined under with a Nikon Eclipse E400 microscope under brightfield illumination, and photographs of sections were made with a Kodak DC120 Digital Camera.

Plate 1, Figures A, B and C, (Appendix 7.3) show photomicrographs of the normal histology of the mouse ankle joint. It should be noted that the normal joint space is lined with a thin layer of synovium overlaying the cartilaginous layer. Deep to the cartilage is hard bone, which in these decalcified sections, appears as an eosinophilic matrix. In the lower power view (Plate 1, A) one can appreciate the bone marrow and extensor tendons. The synovium is relatively acellular and in the high power view can be seen at the corner of the joint space.

In contrast to the normal histology of the mouse joint, the arthritic mouse joint shown in Plate 1, Figures D, E and F, demonstrate the characteristic signs of inflammation. The normal architecture of the cartilaginous layer has been disrupted with a locus of intense inflammation, characterized by the presence of macrophages, neutrophils, and fibroblasts. One can appreciate that the inflammation has caused a near total erosion of the cartilaginous layer of the joint, and the erosion has extended into the bone and approaches the marrow cavity (see Figure E arrow). In addition, there is evidence of an intense inflammatory synovitis (see Figure D arrow).

These changes are consistent with those seen in human RA. It should be noted that in the group of mice that received CM, some animals developed arthritis and some showed no clinical signs. We could not distinguish between an animal that did not develop RA due to the effect of CM from the animals that simply did not develop arthritis (only 70-75% of mice immunized with bovine type II collagen develop arthritis). Histopathology does not, in my opinion, represent a useful clinical measure of the anti-arthritic effect of CM, but it does provide a strong indication that the disease model is developing appropriately.

5.2.5 Conclusion

It appears that when compared with the PBS control, both 450 mg/kg and 900 mg/kg dosages of CM reduced both the frequency (number of mice that showed signs) and severity (maximum severity score; a measure of inflammation) of collagen-induced arthritis in DBA/1LacJ mice. The

difference seen in the mice treated with 450 mg/kg was significantly different from the PBS control at the 95% confidence level.

5.3 Second Collagen-Induced Arthritis Study in DBA1/LacJ Mice

The previous study was designed to verify the earlier work of Diehl and May (1980), and involved the parenteral administration of CM in very high dosages (450 and 900 mg/kg). In the present study, a dosage equivalent to the amount of CM in 12 capsules of AlphaFlex™ (21.4 mg/kg) was given to the mice on a daily basis throughout the course of the experiment. The CM was absorbed onto a 1 mg food pellet, and each animal was fed one CM-treated or control pellet each day prior to *ad libitum* feeding of normal mouse chow. Because the mice were deprived of food between 7 pm and 8 am to facilitate the operant training (see below), it was very easy to get the mice to consume the pellet as the first food presented after their overnight fast.

5.3.1 Study Design

Cage	Mouse #	Arthritis	Treatment	Operant Training
1	A1-5	+	CM	+
2	A6-9	+	CM	+
3	A10-14	+	P	+
4	A15-18	+	P	+
5	A19-21	-	CM	+
6	A22-26	+	CM	-
7	A27-30	+	P	-

CM = cetyl myristoleate (21.4 mg/kg orally)

P = placebo

The study consisted of 30 DBA/1LacJ mice, 27 of which were injected with bovine type II collagen as described above. In a slight variation from the procedure in study two, the mice were given a booster immunization with type II collagen in Freund's incomplete adjuvant on day 28. Three mice were not immunized, but received CM as a control. Nine operant-trained and 5 untrained mice were given the daily oral CM treatment, while 9 operant-trained and 4 untrained mice were given placebo (a food pellet without CM). Three mice were given the CM treatment, but were not immunized with type II collagen. The mice were observed clinically for signs of arthritis just as described in the first study.

5.3.2 Operant Training

In the initial antibody-induced arthritis study, we looked at two operant training regimens; lever press and topography. For the present study, we chose to train the mice in the topography regimen which, as described above, consisted of a shaped movement behavior in which the subject mouse was trained to turn left from the food hopper, left again and down the wall to the rear of the chamber, and left again to the far corner of the chamber. This consisted of 13 shaping steps and resulted in a highly regimented movement pattern. Three mice were not given the arthritis immunization and served as controls for normal movement behavior. However, because the movement pattern of each mouse was established prior to the induction of arthritis, each subject mouse served as its own internal control.

5.3.3 Clinical Results of the Study

The summary clinical data are given in Appendix 7.2. Although the mice in this study were given a booster immunization of type II collagen in IFA on day 28, the progression of the disease was slower and of less magnitude (ie., no maximum severity scores > 4) than was seen in the first collagen-induced arthritis study in which mice were only given the initial type II collagen immunization in CFA. It is possible that the type II collagen immunogen was partially denatured during the preparation process, or that the batch of CFA used was less potent. Nevertheless, the mice did present with clinical signs of arthritis that allowed us to score the progression of the disease. In the placebo group, 65% of the mice showed some signs of arthritis (independent of severity), whereas only 36% of the CM-treated mice showed any signs of arthritis. We elected to plot the average severity score over time to show the progression of arthritis, and these data are shown in Figure 5.3.3.1 and Figure 5.3.3.2. It can be seen that beginning on day 32, and more prominently on days 36 and 42, the placebo control mice had higher average severity scores than the mice given daily oral doses of 21.4 mg/kg CM. Although the differences between the means of these severity scores did not achieve significance at the usual 95% confidence level, the day 42 results were significant at the 90% confidence level (one-tailed t test). In addition, when the maximum severity score for each mouse was determined, there was a difference between the CM-treated mice and placebo controls significant at the 0.13 level of probability. It should also be pointed out that two of the placebo mice showed signs of systemic inflammatory disease and died on days 43 and 44 respectively. No CM-treated mice showed any signs of systemic inflammatory disease.

Figure 5.3.3.1.

As in the previous

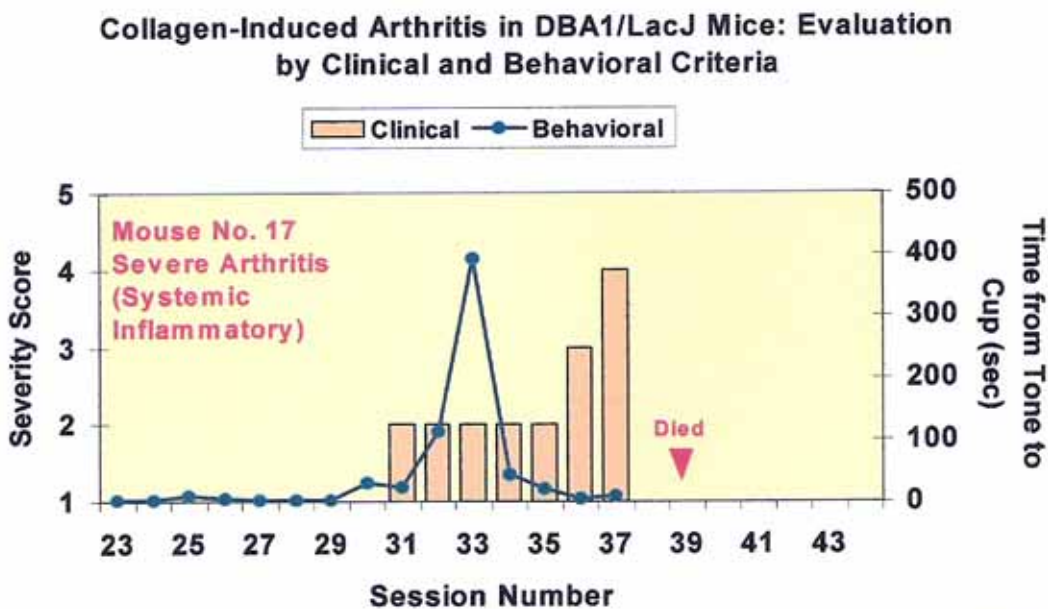
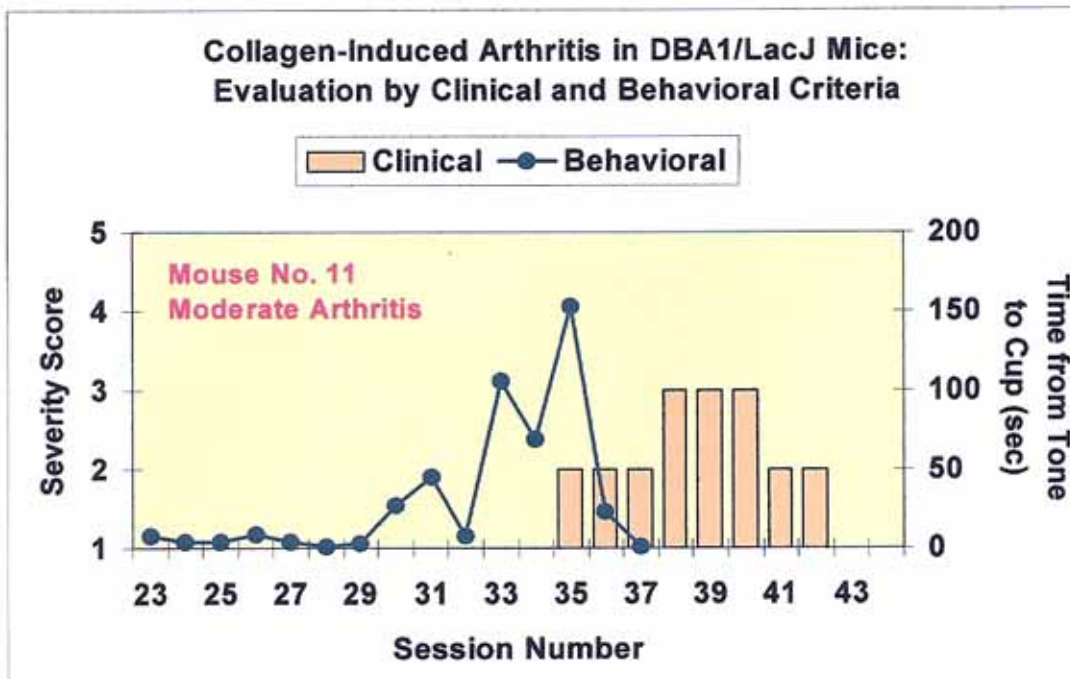


Figure 5.3.3.2

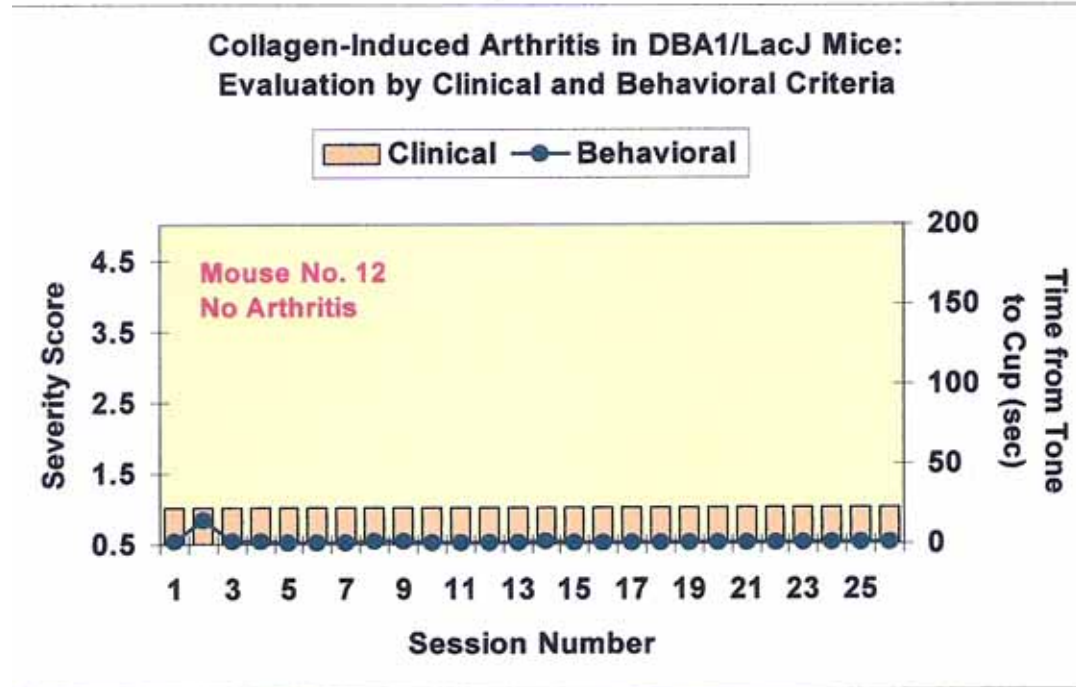


experiment, weights of the mice were measured daily and indicated that the operant trained mice on the food deprivation regimen gained weight through out the course of the experiment. Once again, this is consistent with published results in operant training and would not affect the results of the arthritis study.

5. Conclusion

A lower percentage (36%) of CM-treated mice developed any signs of arthritis as compared with the placebo-treated controls (64%). The severity of the arthritis was also less in the CM-treated vs. placebo mice. It can be concluded that daily oral administration of CM at 21.4 mg/kg (equivalent to 12 capsules of AlphaFlex™) provided some protection against collagen-induced arthritis in

Figure 5.3.3.3.



DBA1/LacJ mice.

6. Bibliography

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7. Appendices

7.1 First Collagen-Induced Arthritis Study: Clinical Severity Scores

Observations for Day 21 (Friday, January 19, 2001)

PBS Control			450 mg/kb CM			900 mg/kb CM		
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1	-	21	1	-
2	1	-	12	1	-	22	1	-
3	1	-	13	1	-	23	1	-
4	1	-	14	1	-	24	1	-
5	1	-	15	1	-	25	1	-
6	1	-	16	1	-	26	1	-
7	1	-	17	1	-	27	1	-
8	1	-	18	1	-	28	1	-
9	1	-	19	1	-	29	1	-
10	1	-	20	1	-	30	1	-

Observations for Day 28 (Friday, January 26, 2001)

PBS Control			450 mg/kb CM			900 mg/kb CM		
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1	-	21	1	-
2	1	-	12	1	-	22	1	-

3	1	-	13	1	-	23	1	-
4	2	lfa	14	1	-	24	1	-
5	2	lfa/rfa	15	1	-	25	1	-
6	2	lfak/rfa	16	1	-	26	1	-
7	1	-	17	1	-	27	1	-
8	1	-	18	1	-	28	1	-
9	1	-	19	1	-	29	1	-
10	1	-	20	2	lfa/rfak	30	1	-

Observations for Day 21 (Saturday, February 3, 2001)

PBS Control			450 mg/kb CM			900 mg/kb CM		
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1	-	21	2	rfa
2	1	-	12	1	-	22	2	lfa
3	1	-	13	1	-	23	1	-
4	2	lfa	14	1	-	24	1	-
5	2	lfa	15	1	-	25	1	-
6	3	lfak/rfa	16	1	-	26	1	-
7	1	-	17	1	-	27	1	-
8	1	-	18	1	-	28	2	lfa
9	1	-	19	1	-	29	1	-
10	1	-	20	3	lfak/rfa	30	1	-

Observations for Day 28 (Saturday, February 10, 2001)

PBS Control			450 mg/kb CM			900 mg/kb CM		
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1	-	21	2	rfa
2	1	-	12	1	-	22	3	lfak
3	1	-	13	1	-	23	1	-
4	5	lfak	14	2	lfa	24	1	-
5	5	lfak/rfak	15	1	-	25	1	-
6	4	lfak/rfak	16	1	-	26	1	-
7	1	rfa	17	1	-	27	1	-
8	1	-	18	1	-	28	3	lfak
9	1	rfa	19	1	-	29	1	-
10	1	rfa	20	4	lfak/rfak	30	2	rfa

Observations for Day 57 (Saturday, February 24, 2001)

PBS Control			450 mg/kb CM			900 mg/kb CM		
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1	-	21	1 ↓	-
2	1	-	12	1	-	22	2 ↓	lfa
3	1	-	13	1	-	23	1	-
4	5	lfak	14	2	↓	24	1	-
5	5	lfak/rfak	15	1	-	25	1	-

6	5	lfak/rfak	16	1	la	26	1	-
7	3	rfak	17	1	-	27	1	-
8	1	-	18	1	-	28	2 ↓	lfa
9	1	↓	19	1	-	29	1	-
10	2	rfa ↓	20	4	lfak/rfak	30	1 ↓	-

- lfak = inflammation of left foot, ankle, knee
↓ = decrease from previous observation

7.2 Second Collagen-Induced Arthritis Study: Clinical Severity Scores

7.2.1 Summary Data and Statistical Analysis

Cetyl Myristoleate-Treated Mice

Day/Mouse	1	2	3	4	5	6	7	8	9	22	23	24	25	26	Mean ±SD	p value*
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	n/a
32	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1.07±0.27	n/a
35	1	2	1	1	1	1	1	2	1	1	1	2	1	1	1.27±0.45	0.34
38	1	2	1	1	2	1	1	2	1	1	1	2	1	1	1.29±0.47	0.14
42	1	2	1	1	3	1	1	2	1	2	1	2	1	1	1.43±0.65	0.10
46	1	2	1	1	3	1	1	2	1	2	1	3	1	1	1.5±0.76	0.60
49	1	1	1	1	2	1	1	2	1	1	1	2	1	1	1.21±0.43	0.55
Max Score	1	2	1	1	3	1	1	2	1	2	1	3	1	1	1.5±0.76	0.13

* one tailed Student's t test for unpaired samples, CM=treated vs. Placebo on the same day

Placebo-Treated Mice

Day/Mouse	10	11	12	13	14	15	16	17	18	27	28	29	30	Mean ±SD	p value*
28	1	1	1	1	1	1	D	1	D	1	1	1	1	1	n/a
32	1	1	1	1	1	2		2		1	1	1	1	1.09±0.30	n/a
35	1	2	1	2	2	2		2		2	1	1	1	1.45±0.52	0.34
38	1	3	1	2	2	2		3		2	1	1	1	1.64±0.67	0.14
42	1	2	1	2	2	4		4‡		2	1	2	1	2.0±1.0	0.10
46	1	1	1	2	2	D		D		2	1	2	1	1.67±0.71	0.60
49	1	1	1	1	2					2	1	1	1	1.33±0.5	0.55
Max Score	1	3	1	2	2	4	4	4		2	1	2	1	2.09±1.14	0.13

* one-tailed Student's t test for unpaired samples, CM=treated vs. Placebo on the same day

‡ this mouse attained a severity score of 4 before death on day ⁴³

7.2.2 Clinical Observations

7.2.1 Summary Data and Statistical Analysis

Observations for Day 28 (Wednesday, April 18, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1		21	1	
2	1	-	12	1		22	1	
3	1	-	13	1		23	1	
4	1	-	14	1		24	1	
5	1	-	15	1		25	1	
6	1	-	16	dead*		26	1	
7	1	-	17	1		27	1	
8	1	-	18	dead*		28	1	
9	1	-	19	1		29	1	
10	1	-	20	1		30	1	

* Died on 4/17/01 during administration of booster immunization

Observations for Day 32 (Sunday, April 22, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1		21	1	
2	2	lp	12	1		22	1	
3	1		13	1		23	1	
4	1		14	1		24	1	
5	1		15	2	rfa	25	1	
6	1		16			26	1	
7	1		17	2		27	1	
8	1		18			28	1	
9	1		19	1		29	1	
10	1		20	1		30	1	

Observations for Day 35 (Wednesday, April 25, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	2	-	21	1	-
2	2	lp	12	1	-	22	1	-
3	1	-	13	2	rfa	23	1	-
4	1	-	14	2	rfa	24	1	rfa
5	2	-	15	2	rfa	25	1	-
6	1	-	16		-	26	1	-
7	1	-	17	2	rfa	27	1	rfa
8	2	lp	18		-	28	1	-
9	1	-	19	1	-	29	1	-
10	1	-	20	1	-	30	1	-

Observations for Day 38 (Saturday, April 28, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	3	rfa	21	1	-
2	2	lp	12	1	-	22	1	-
3	1	-	13	2	rfa	23	1	-
4	1	-	14	2	lfa	24	2	lfa
5	2	rfa	15	2	rfa	25	1	-
6	1	-	16		-	26	1	-
7	1	-	17	3	rfa	27	2	lfa
8	2	lp	18		-	28	1	-
9	1	-	19	1	-	29	1	-
10	1	-	20	1	-	30	1	-

Observations for Day 42 (Wednesday, May 2, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1		11	2	rfa	21	1	
2	2	lp	12	1		22	2	rp
3	1		13	2	rfa	23	1	
4	1		14	2	lfa	24	2	lfp
5	3	rp, rfa	15	4 *	rfa, rp	25	1	
6	1		16			26	1	
7	1		17	4 *	rfa	27	2	lfa
8	2	lp	18			28	1	
9	1		19	1		29	2	rp
10	1		20	1		30	1	

* These mice were moribund, with signs of systemic inflammatory disease

Observations for Day 38 (Saturday, April 28, 2001)

Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
1	1	-	11	1 ↓	-	21	1	-
2	2	lp	12	1	-	22	1	rp
3	1	-	13	2	rfa	23	1	-
4	1	-	14	2	lfa	24	2	lfp
5	3	rp, rfa	15	dead *	rfa, rp	25	1	-
6	1	-	16			26	1	-
7	1	-	17	dead **	rfa	27	2	lfa
8	2	lp	18		-	28	1	-
9	1	-	19	1	-	29	1	rp
10	1	-	20	1	-	30	1	-

* Died on 5/3/01 of systemic inflammatory disease

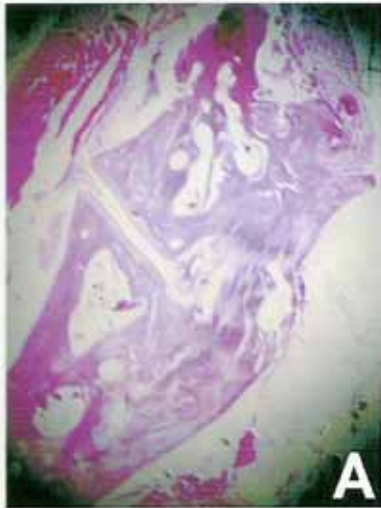
** Died on 5/4/01 of system inflammatory disease (mouse was moribund, with max. severity score of 4 – rfa)

Observations for Day 49 (Wednesday, May 9, 2001)

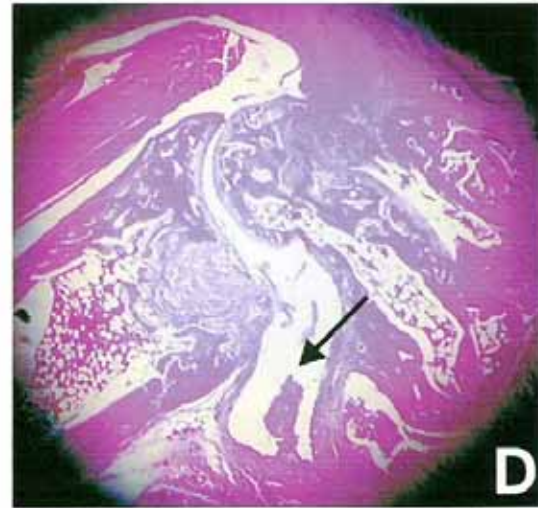
Mouse #	Score	Observ	Mouse #	Score	Observ	Mouse #	Score	Observ
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2	1 ↓ *	-	12	1	-	22	1 ↓	-
3	1	-	13	1 ↓	-	23	1	-
4	1	-	14	2	lfa	24	2	lfp
5	2 ↓	rp, rfa	15	dead *	rfa, rp	25	1	-
6	1	-	16		-	26	1	-
7	1	-	17	dead **	rfa	27	2	lfa
8	2	lp	18		-	28	1	-
9	1	-	19	1	-	29	1	-
10	1	-	20	1	-	30	1	-

* lfak = inflammation of left foot, ankle, knee

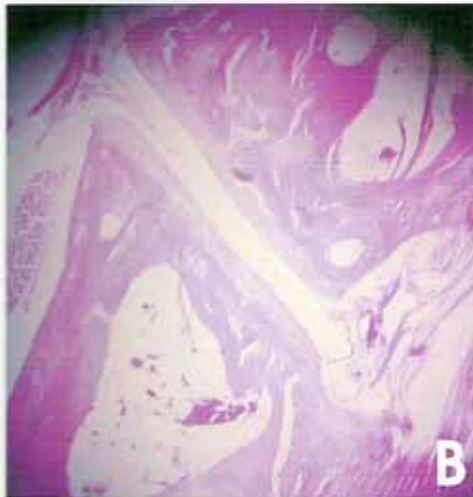
7.3 Histopathology Photomicrographs



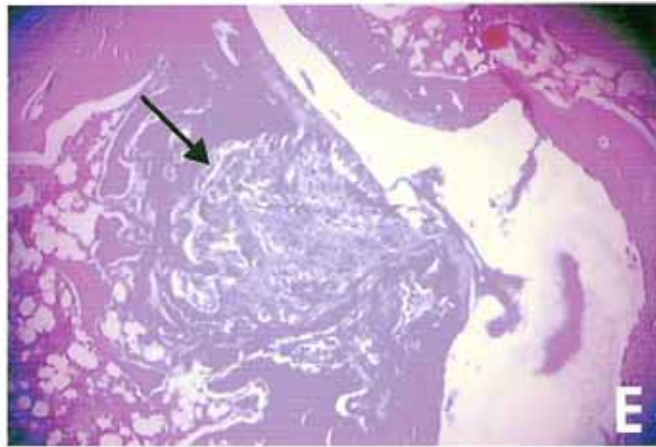
Normal ankle joint. 4X



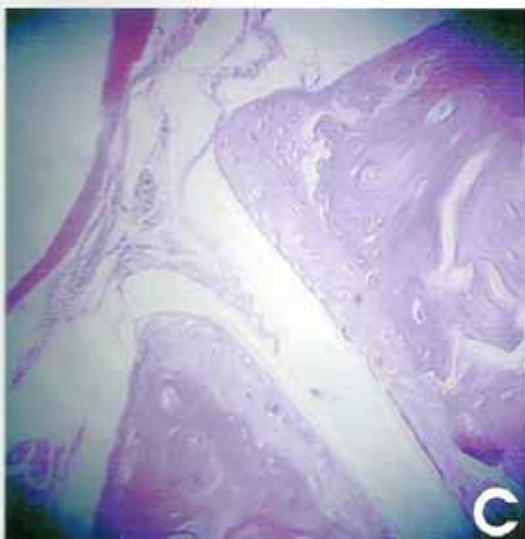
Arthritic ankle joint. 4X



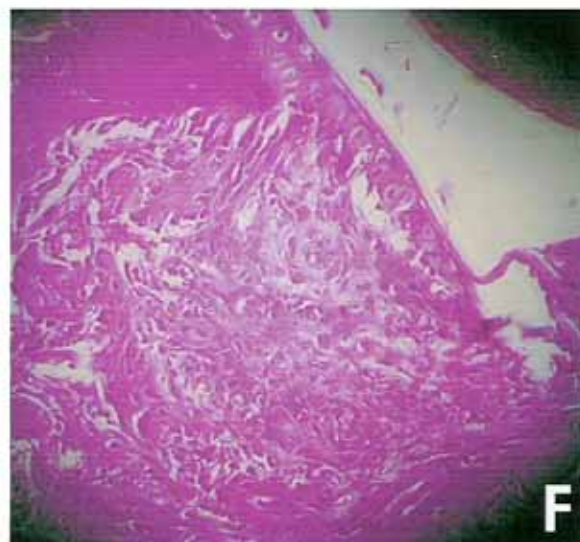
Normal ankle joint. 10X



Arthritic ankle joint. 10X



Normal ankle joint. 20X



Arthritic ankle joint. 20X