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The Effects of 13-cis Retinoic Acid on Cytokeratin Expression in MDA-886Ln Monolayer Cell Culture: Immunohistochemical Analysis and Correlation to Cellular Autofluorescent Patterns

Michelle R. Yagoda, M.D.*
Howard E. Savage, Ph.D.*
Peter G. Sacks, Ph.D.*
Pilar Garin-Chesa, M.D., Ph.D.**
T.J. Yao, Ph.D.***
Philip J. LoPresti, M.D.*
Stimson P. Schantz, M.D.*

*Head and Neck Service, Department of Surgery; Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, N.Y., N.Y. 10021
**Department of Experimental Pathology, Ludwig Institute; Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, N.Y., N.Y. 10021
***Department of Epidemiology and Biostatistics; Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, N.Y., N.Y. 10021

ABSTRACT

Objective - Antitumor therapy which focuses on indiscriminant destruction of all dividing cells has met with limited success. Whereas, neoplastic transformation is characterized by abnormal cellular differentiation; an alternate method of antitumor therapy may be to change the differentiation status of malignant cells with retinoids. Since differentiation status can be identified by cytokeratin expression patterns and malignant cells can be distinguished from normal cells non-invasively by autofluorescence, we aim to correlate alterations in cytokeratin expression with variations in cellular autofluorescence.

Design - MDA886Ln Squamous Cell Carcinoma cells were grown in monolayer cell culture as these cells constitute a model in which retinoid sensitivity has been well-documented. An experimental group was treated with [10^{-6}] M 13-cis retinoic acid for three, five and seven days. A control group was also maintained. An equal number of cells were taken from the control and experimental groups and were submitted to both monoclonal antibody immunohistochemical analysis using the Avidin-Biotin-Complex technique and to cellular autofluorescence, a non-invasive technique to detect differences in spectral patterns presumably representing subcellular biochemical changes.

Results - Cytokeratin 19 expression, as detected by immunohistochemistry, was significantly increased in three separate trials after seven days of exposure to 13-cis retinoic acid. A significant increase in intensity is noted in the emission scan (EX365, λ400-700nm) for the experimental group. Additionally, the ratio of intensities of the peaks at 270 nm and 290nm in the excitation scan (EM380,λ 220-360nm) was noted to decrease significantly in the experimental group.

Conclusion - 13-cis retinoic acid enhances cytokeratin 19 expression after seven days of exposure in monolayer cell culture. This represents promotion of differentiation and correlates to a significant increase in peak intensity of the EX365 scan and a decrease in peak ratios in the EM380 scan. Thus, there is a correlation between cytokeratin expression and cellular autofluorescence patterns.

BACKGROUND/INTRODUCTION:

The capacity to identify subclinical neoplastic disease of the upper aerodigestive tract without invasive biopsy would greatly facilitate cancer detection strategies. One manner in which to achieve this end lies within the concept of cell autofluorescence. Fluorescence spectroscopy based on the principles of optical spectroscopy and laser technology employs extrinsic fluorescent chromophores to characterize the physiologic state of a tissue. It has been in use for over a half century.[1] However, autofluorescence spectroscopy, introduced for clinical application in 1981 by Alfano, differs in that it uses intrinsic fluorophores naturally present in cells[2,3,4] Its use to date has been numerous and specifically of interest in that it has enabled the distinction of benign and malignant tissue using the ratio of fluorescent intensities at specific wavelengths.[5,6,7,8]

Currently, antitumor therapy which focuses on indiscriminantly destroying all dividing cells has met with limited success. An alternate method may be to non-invasively identify malignant cells and alter their differentiation status. With that in mind, we proposed the following hypothesis: 13 cis-retinoic acid (13-cis RA) induced changes in cell differentiation can be detected non-invasively by cellular autofluorescence and correlated to changes in cytokeratin expression.

The bases of this study are that neoplastic transformation can be characterized by abnormal cellular differentiation; that antitumor therapy with retinoids can alter cell differentiation status[9]; that differentiation status can be evaluated by cytokeratin expression patterns[10,11]; and, that changes in cytokeratin expression can be correlated to variations in cellular autofluorescence.

We will use *in vitro* cellular models to develop this relationship. We will treat MDA886Ln squamous cell carcinoma cells with13 cis-RA, an agent known to modulate cell differentiation. [12,13] These cells comprise a model in which retinoid sensitivity has been well-documented. We have already shown that there are detectable changes in native cellular fluorescence patterns after treatment with retinoids. We propose that retinoid-induced effects represent changes in epithelial differentiation status which will be detected by variations in cytokeratin subclass expression. Cytokeratins (CK), subcellular level biomarkers of epithelial differentiation, can be detected by immunohistochemical analysis and may prove to be intermediate endpoint biomarkers of malignant transformation.[14] We will demonstrate that cellular differentiation characteristics can be modulated with retinoids, detected by cellular autofluorescence and correlated to patterns of cytokeratin expression.

The purposes of this study are 1) to detect time-dependent spectral changes in cells treated with 13 cis-RA; 2) to show that these fluorescent changes are independent of cell number (i.e. proliferation); 3) to identify retinoid induced changes in cytokeratin expression (i.e. differentiation); and, 4) to correlate cytokeratin expression and autofluorescent spectral patterns with changes in differentiation status.

MATERIALS AND METHODS:

Cell Culture

MDA-886Ln cells derived from a lymph node metastasis of a T_3N_{3a} Squamous Cell Carcinoma of the larynx in a sixty-four year-old male were chosen for monolayer cell culture as sensitivity to retinoids were previously well-documented. 13 cis-retinoic acid at $[10^{-6}]$M was selected as a modifier of cell differentiation as previous dose-response experiments proved this concentration to be effective.[15] Monolayer cell culture was performed in 1:1 Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12), supplemented with 10% Fetal Calf Serum and 50µg/ml gentamycin. Cells were incubated at 37° C and in 5% CO_2.

On day #0, the experimental group received 13 cis-retinoic acid at $[10^{-6}]$M. The control group was administered media (DMEM/F12) with the control vehicle (100% EtOH) only. Media was changed every 48-72 hours. Cells were evaluated after three, five and seven days of treatment.

On the day of evaluation, cells were subjected to 0.125% trypsin/0.125% EDTA in PBS without calcium or magnesium and collected. Cell count was performed via Coulter Counter. A growth curve was prepared to verify the efficacy of 13 cis-RA. The experiment was conducted three times for statistical purposes, and cell counts were used as internal controls between trials. On days three, five and seven, an aggregate of cells (7×10^6) from the experimental group and an equal number of cells from the control group were obtained for cell autofluorescence. Additionally, cells (5×10^5) from each group were set aside for immunohistochemistry.

Autofluorescence:

Volumes of equal number of cells (7×10^6) were placed into two centrifuge tubes and subjected to 1000rpm for five minutes at 18° C. The supernatant was removed, the cells were resuspended in PBS without calcium or magnesium and centrifugation was again performed. This step was repeated a second time. After the supernatant was discarded, a fibrin clot was made from the remaining pellet of cells. [Previous work by Dr. Zhang demonstrated that a fibrin clot was instrumental in reducing the number of cells needed to provide ample volume for spectroscopy and that the fibrin clot did not interfere with the data obtained by autofluorescence over the range of scans performed.][16] Twenty µl of thrombin (SIGMA Chemicals, T-7513, #76F-9465) was added to the pellet of cells. Then, 10 µl of fibrinogen (SIGMA Chemicals, F-4753, #55F-9305) was added to the suspension. This mixture was placed into a special dental mold (made by Dr. Zhang) and incubated for twenty minutes at 37° C. This gel-like clot provided for a columnar volume of cells which was of the ideal shape and size for spectroscopy. Without distortion of

shape, the clot was carefully placed into a quartz cuvette specifically designed for the Mediscience Technology CD Scan Equipment and subjected to autofluorescence.[17]

Two types of scans were performed: excitation scans - those in which the wavelength of the exciting beam is varied and the emission at a given wavelength is recorded; and, emission scans - those in which the excitation wavelength is held constant and a range of emission wavelengths are evaluated.

Immunohistochemistry:

Volumes of equal number of cells (5×10^5) were placed into microcentrifuge tubes and subjected to 1000rpm for five minutes and 18° C. The supernatant was removed and the cells were resuspended in one milliliter of serum-free media (DMEM/F12). Cytospin (800 rpm for five minutes) was performed in order to deposit 5×10^4 cells on each poly-L-lysine coated slide. Cells were then lightly fixed in 95% alcohol and stored at -70° C for later immunohistochemical analysis.

Immunohistochemistry was performed using the Avidin-Biotin-Complex (ABC: Vector Laboratories, 1:1 ratio at 1:100) method. The cytokeratin expression pattern of the MDA-886Ln cell line was unknown; therefore, we chose to evaluate CK10 (suprabasal, cornified, stratified); CK13 (suprabasal, non-cornified, stratified); CK14 (basal) and CK19 (simple/malignant). Additionally, we chose CK AE1 a broad spectrum antibody. Antibodies used were the IgG1 based cytokeratin 10, 13, 19, AE1 and the IgM based cytokeratin 14. (SIGMA Immunohistochemicals, St. Louis, MO, C-7284, C-0791, C-7159, C-8791). AE1 was supplied by Dr. Tung Tien Sun of the Department of Dermatology who provided the α-cytokeratin hybridoma AE1 which was processed and later used to collect ascites. The ascites was purified using Protein A. Negative controls were IgG1 for cytokeratins 10, 13, 19 and AE1; and PBS for cytokeratin 14.

Cells were thawed for thirty minutes, then washed twice in PBS. Non-immune suppressor serum of the same host animal was used for ten minutes to block endogenous peroxidase activity and non-specific binding. Cells were again washed twice in PBS. The primary antibodies were applied in the previously titrated concentrations and incubated overnight. [AE1 (5μg/ml),CK10 (1:40), CK13 (1:250),CK14 (1:400), CK19 (1:50)] The cells were again washed twice in PBS and the secondary antibody was applied for thirty minutes. The cells were washed twice in PBS and the tertiary antibody was applied for thirty minutes. Finally, the cells were washed in PBS, stained with DAB, and counter-stained with Hematoxylin.

Slides were scored blind by the first author (MY), on the following scale: 0=negative; 1=1-25%; 2=26-50%; 3=51-75%; and, 4=76-100%. Those slides with identical scores between experimental and control groups were reevaluted. and assigned a "+" to those in which there was an identifiable change in staining

(ie, several positive cells to 20%) which was not otherwise reflected in the scoring.

RESULTS:

Growth Curve:

An experimental group of 2×10^4 cells/ ml were treated with [10^{-6}] M 13-cis retinoic acid. A control group of 2×10^4 cells/ ml were treated with vehicle alone. A growth curve was established which is seen in Figure 1 and is based on the data from Table I. 13-cis retinoic acid's effect on inhibition of cell growth, as evidenced by cell count, was noted to begin on day three of treatment and increase with time.

Immunohistochemistry:

The labelling indices are depicted in Table II. Cytokeratin 19 expression as seen in Figure 2, was noted to increase as early as three days after exposure to 13-cis retinoic acid. Significance was achieved after seven days (Figure 3) of treatment. ($p<0.01$; one way signed rank analysis)

Cytokeratin 13 expression was variable after treatment with 13-cis retinoic acid for three, five, and seven days. The expression of AE1 and cytokeratin 10 demonstrated a trend toward increased expression but this did not achieve significance. Cytokeratin 14 was not present in the control group; nor was its expression induced by treatment with 13-cis retinoic acid.

Autofluorescence:

Significant time dependent spectral changes were not seen in cells treated with 13-cis retinoic acid prior to day seven of treatment. The changes seen on day seven between control and experimental groups were independent of cell number. No spectral interference from the fibrin clot was noted as seen in Figure 4.

The excitation spectra for the emission scan EX365 (λ400-700nm) for control and experimental groups is seen in Figure 5. The peak amplitude is measured at 440 nm and the minimum is taken at 590 nm. At day seven, a significant increase in intensity is noted in the retinoid treated cells ($p<0.03$, T-test with pooled data). Additionally, a trend toward hyperchromatic shift is noted the 440-590 nm range after seven days of treatment.

The emission spectra for the excitation scan EM380 (λ220-360nm) for control and experimental groups is seen in Figure 6. To quantify analysis, we evaluated spectral changes observed in the 270/290nm peak intensity ratios. A significant decrease in this ratio was noted for the experimental group after seven days ($p<0.01$, T-test with paired data). An increase in the intensities at 270nm and 290 nm for the experimental group after seven days of retinoid

exposure approached significance ($p<0.07$, T-test with pooled data). A trend toward hypochromatic shift was also noted in the treated group in the 220-290nm range.

STATISTICAL ANALYSIS:

One way signed rank test, Mann-Whitney confidence interval test, Mantel-Haenszel chi-square test and T-tests for pooled and paired data was employed using the SAS system and the Minitab program.

DISCUSSION:

The capacity to identify subclinical neoplastic disease of the upper aerodigestive tract without invasive biopsy (using autofluorescence) can significantly facilitate cancer detection strategies.[18,19] Additionally, non-invasive *in vivo* surveillance monitoring the effects of chemotherapeutic agents would be of great benefit. In this study, we use the MDA886Ln SCCA line and 13-cis retinoic acid as a well-documented model system[20] in which we can test our hypothesis: 13-cis retinoic acid induced changes in cell differentiation can be detected non-invasively by autofluorescence and correlated to changes in cytokeratin expression.

Vitamin A (retinol) and the family of retinoid derivatives are essential for normal epithelial differentiation.[21,22] Vitamin A deficiency in rats results in failure of stem cells to differentiate into mature epithelial cells.[23] Whereas, normal stem cells divide into terminally senescent cells; cancer cells divide but fail to differentiate.[24]

Although retinoid derivatives are not equally effective and resistance may develop, continued presence of selected agents can achieve an on-going chemopreventive or chemotherapeutic effect.[25,26,27,28,29,30] *In vitro* effectiveness in inhibition of tumor growth has been acceptably measured by cell count.[31,32,33] *In vivo* administration has been well-tolerated and well-documented to modulate cell differentiation and induce embryonic phenotype differentiation in keratinocytes.[34,35]

Retinoids have been documented to modulate cell differentiation through RA receptors and cellular retinoid binding proteins which transport RA from the cytosol to the nucleus where RA exerts its effects on chromatin and gene expression.[36,37] cAMP modulates the effects of RA by altering expression of the RA receptors by post-transcriptional mechanisms and modifying membrane ion-channel activity.[38,39] Through its effects on ion-channel activity, RA can lower calcium levels which has been shown to reversibly affect proliferation, differentiation and migration of keratinocytes *in vitro*.[40] Through kinases, RA modulates the activity and expression of various oncogenes, resulting in irreversible alteration of cell phenotypes.[41] The ultimate effects on cell differentiation are varied - terminal differentiation may be inhibited in some cases and promoted in others.[42]

The mechanism by which RA modulates differentiation is unclear. We propose an explanation for this apparently unpredictable outcome. Consider that there is a physiologic range of retinol through which normal differentiation proceeds. If a baseline deficiency exists, differentiation can not proceed; hence, cells divide but do not differentiate (as in cancer). In this circumstance, when RA is administered, differentiation is promoted. On the other hand, if the baseline retinol level is physiologic and RA is added to exceed threshold, inhibition of differentiation or abnormal differentiation may occur.

Cytokeratins are polypeptides encoded by a multigene family which include microfilaments (actin), microtubules (tubulin), and intermediate filaments of which there are six classes of intermediate filaments. Keratins are derived from epithelial origin and comprise two of the six classes. The remaining four groups are derived from muscle (desmin), mesenchyme (vimentin), and nerves (neurofilaments). Keratins exist as heteropolymers - a type I acidic subunit coupled with a type II basic or neutral subunit. In malignant transformation, keratin expression has been noted to become uncoupled. Expression is influenced by environmental factors such as inflammation and premaligant transformation; and is dependent on cell type, location and differentiation status.[43]

Cytokeratin Subclasses by Location

	Type I Subunit	Type II Subunit
SUPRABASAL		
cornified, stratified	1,2	9,10,11
cornea	3	12
noncornified,stratified	4	13
fast turnover	6	16
BASAL	5	14,15,17,19
SIMPLE	7,8	18,19,20

Expression of cytokeratins is regulated in part by retinoids.[44,45,46,47] Cytokeratins 13 and 19 have been shown to increase in response to retinoids whereas all other cytokeratins are diminished.[48] In this study, we see a significant increase in cytokeratin 19 expression after exposure to retinoids but only a trend toward increased expression of cytokeratin 13. This may become significant with an increase in sample size, or it may represent disruption of coordinate regulation. Agarwal et al has shown that in retinoid-treated SCC-13 cells, coordinate regulation was uncoupled.[49] The mechanism for gene regulatory uncoupling is not clear, but has been theorized to be a result of retinoid receptor inability to mediate positive transcriptional activation.[50]

At one extreme, expression of cytokeratin 19 may indicate differentiation of stem cells destined to be in simple epithelium or in the basal layer of stratified epithelium; whereas, at the opposite extreme, expression may indicate suprabasal expression indicative of (pre)malignancy. Based on this *in vitro* study, and with the knowledge that 13-cis RA clearly demonstrates chemotherapeutic anc chemopreventive effects *in vivo*, we presume that

increased expression of cytokeratin 19 after exposure to 13-cis RA indicates induced differentiation of undifferentiated MDA886Ln SCCA cells. (It is important not to confuse histologic grading of poor-well differentiated tumors with the terms undifferentiated-terminally differentiated cell types.)

Several differences in autofluorescence spectra between experimental and control groups were noted. Previous work in this lab demonstrated retinoid-induced spectral changes in MTS spheroids after ten days of treatment.[51] These patterns were presumed to be consistent with alterations in cellular electron transport: (↓ NADH), increased expression of aminoacids (↑ flavin and tryptophan); and, increased expression of cytokeratins. Retinoid-induced modulations in cell differentiation staus are correlated to variations in the EM380 and EX365 scans. The exact nature of the biochemical changes detected by cellular autofluorescence is unclear; however, the EX365 scan has been postulated to represent the spectral signature of cytokeratins.[52]

CONCLUSION:

1. 13-cis retinoic acid promotes cytokeratin 19 expression representing induced differentiation;
2. 13-cis retinoic acid-induced changes in differentiation are correlated to variations in the EM380 and EX365 scans; and,
3. retinoid-induced effects are independent of cell number (ie, proliferation)

FUTURE DIRECTIONS:

In order to confirm these results, larger studies need to be conducted encompassing additional cell lines; other agents which modulate differentiation (ie, sodium butyrate); and, additional measures to evaluate differentiation status (involucrin, cornified envelope, RNA/protein analysis). Currently, we are developing a tagged monclonal antibody system to allow for one-step immunohistochemical and autofluorescent analysis.(Schantz et al, in progress) Finally, we are presently separating individual cytokeratins by gel electrophoresis in order to identify individual spectral signatures.(LoPresti et al, in progress) With this data, the nature of the biochemical changes detected by fluorescent patterns may be more definitively identified.

TABLE I

DAY	GROUP	CELLS/MILLILITER
plating	control	21,240
	13-cis RA	28,513
0, RA added	control	21,600
	13-cis RA	28,613
3	control	49,640
	13-cis RA	42,546
5	control	132,216
	13-cis RA	83,566
7	control	211,060
	13-cis RA	100,413

The data from all trials were reproducible to within five percent.

TABLE II

DAY	GROUP	AE1	CK10	CK13	CK14	CK19
3	control	1	2	1	-	3
	13-cis RA	4	4	3	-	4
	control	1	2	2	1	1
	13-cis RA	1/2	2	2	1	3/4
	control	4	4	2/3	-	3/4
	13-cis RA	4	4	4	-	4
5	control	1	3	2	-	1/2
	13-cis RA	3	4	1	-	4
	control	1	1	1	-	1
	13-cis RA	1	1	1	-	1
	control	1	1	1	-	2
	13-cis RA	1+	1	1	-	3
7	control	1	2	1	-	2/3
	13-cis RA	2	2	1+	-	4
	control	1	1	1	-	1
	13-cis RA	1	2	2	-	2/3
	control	3	1	1	-	2
	13-cis RA	2	1	1	-	3/4

KEY
- = 0%
1= 1-25%
2= 26-50%
3= 51-75%
4= 76-100%

TABLE III

SCAN	EXP #	λRA440nm	λRA590nm	λC440nm	λC590nm	λC-RA440[1]	λC-RA590[2]	
EX365	1	38	9	49	10	11	1	
	2	49	11	69	13	20	2	
	3	62	11	74	13	12	2	
SCAN	EXP #	λRA270nm	λRA290nm	λC270nm	λC290nm	λC290/270	λRA290/270	[RA290/270]/[C290/270][3]
EM380	1	313	474	393	646	1.64	1.51	0.13
	2	236	425	259	492	1.90	1.80	0.10
	3	265	487	301	578	1.92	1.84	0.08

*All data is for day seven.

[1] mean=14.33; standard deviation=4.93; standard error of the mean=2.85; $t=5.03$; $p<0.037$

[2] mean=1.67; standard deviation=0.58; standard error of the mean=0.33; $t=5.00$; $p<0.038$

[3] mean=0.10; standard deviation=0.03; standard error of the mean=0.02; $t=7.11$; $p<0.019$

Figure 1

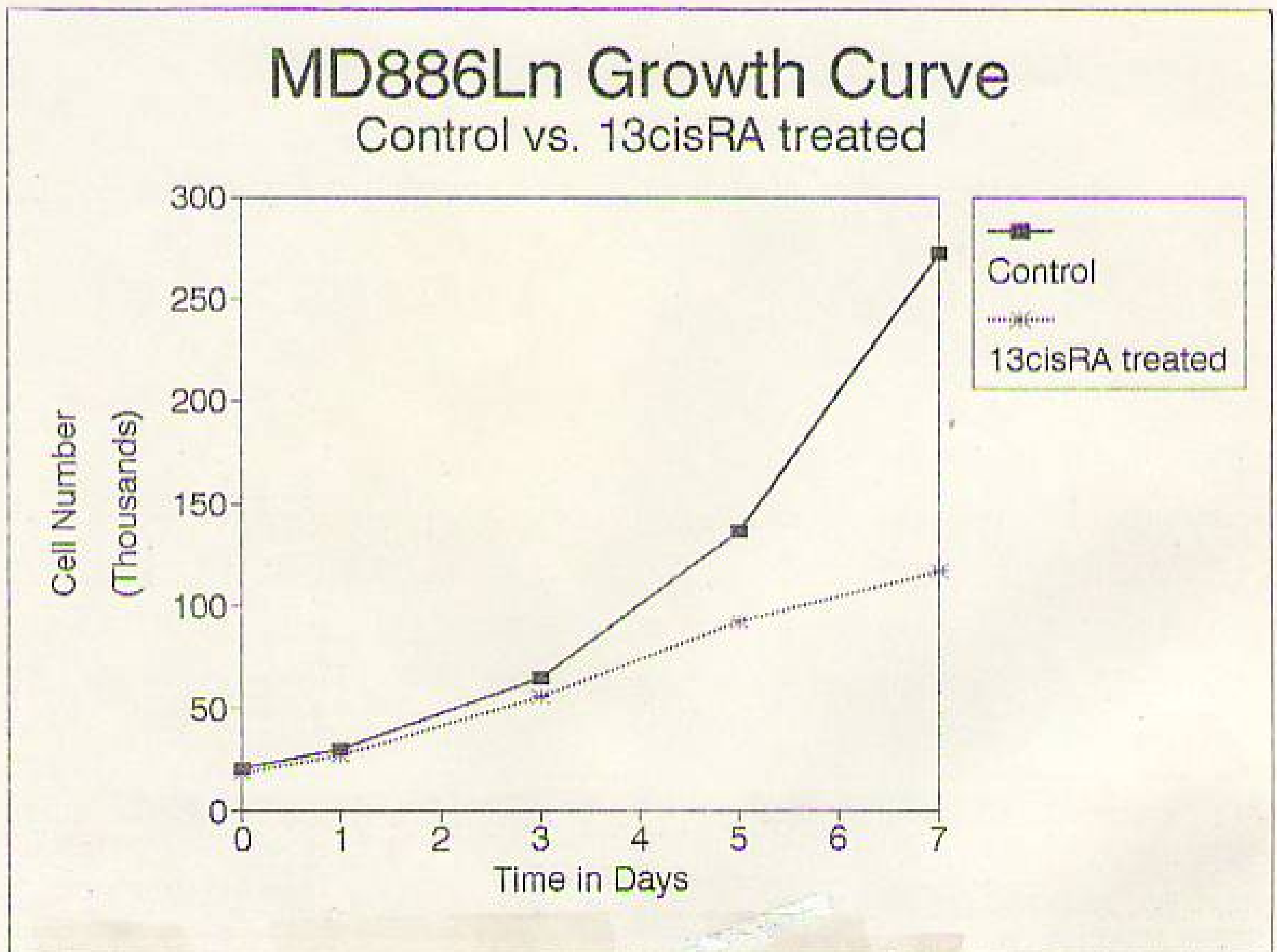
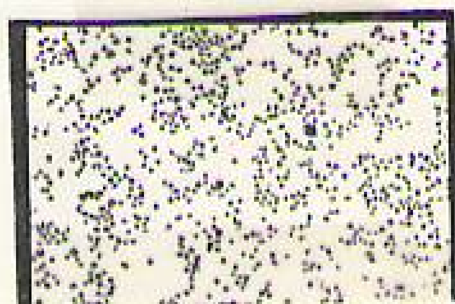


Figure 1. Data from all trials were reproducible to within 5%.

Figure 2



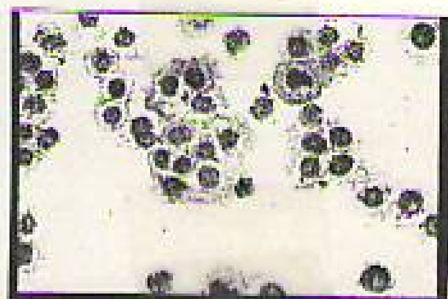
A.

B.

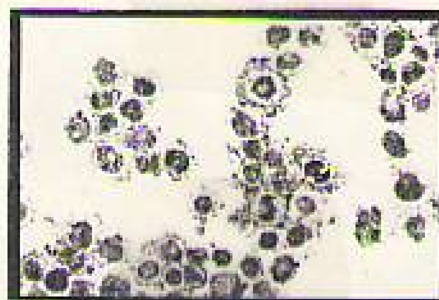
FIGURE 2. CYTOKERATIN 19 EXPRESSION

A. (10X) Control group
B. (10X) 13-cis RA group
Arrows depict cells displaying immunohistochemical staining. Three days of treatment.

Figure 3



A.



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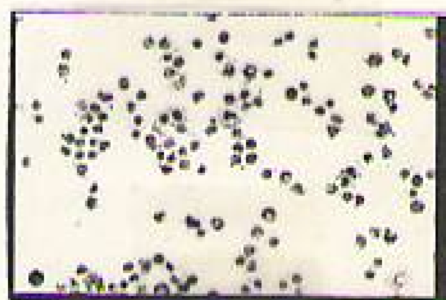
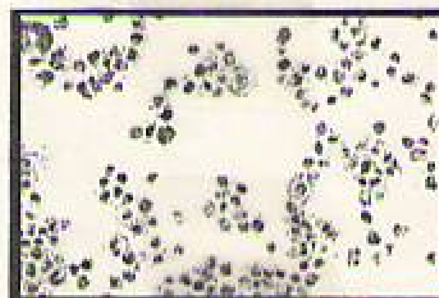
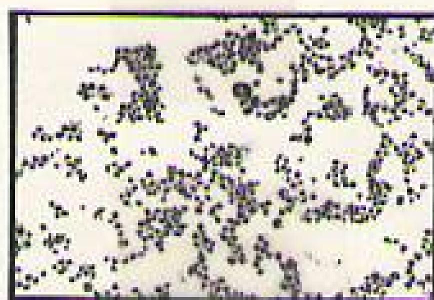


C.



D.

E.



F.

FIGURE 3. CYTOKERATIN 19 EXPRESSION

A. (40X) Control group
B. (40X) 13-cis RA group
C. (20X) Control group
D. (20X) 13-cis RA group
E. (10X) Control group
F. (10X) 13-cis RA group
Arrows depict cells displaying immunohistochemical staining. Seven days of treatment.

<u>Figure 4</u>

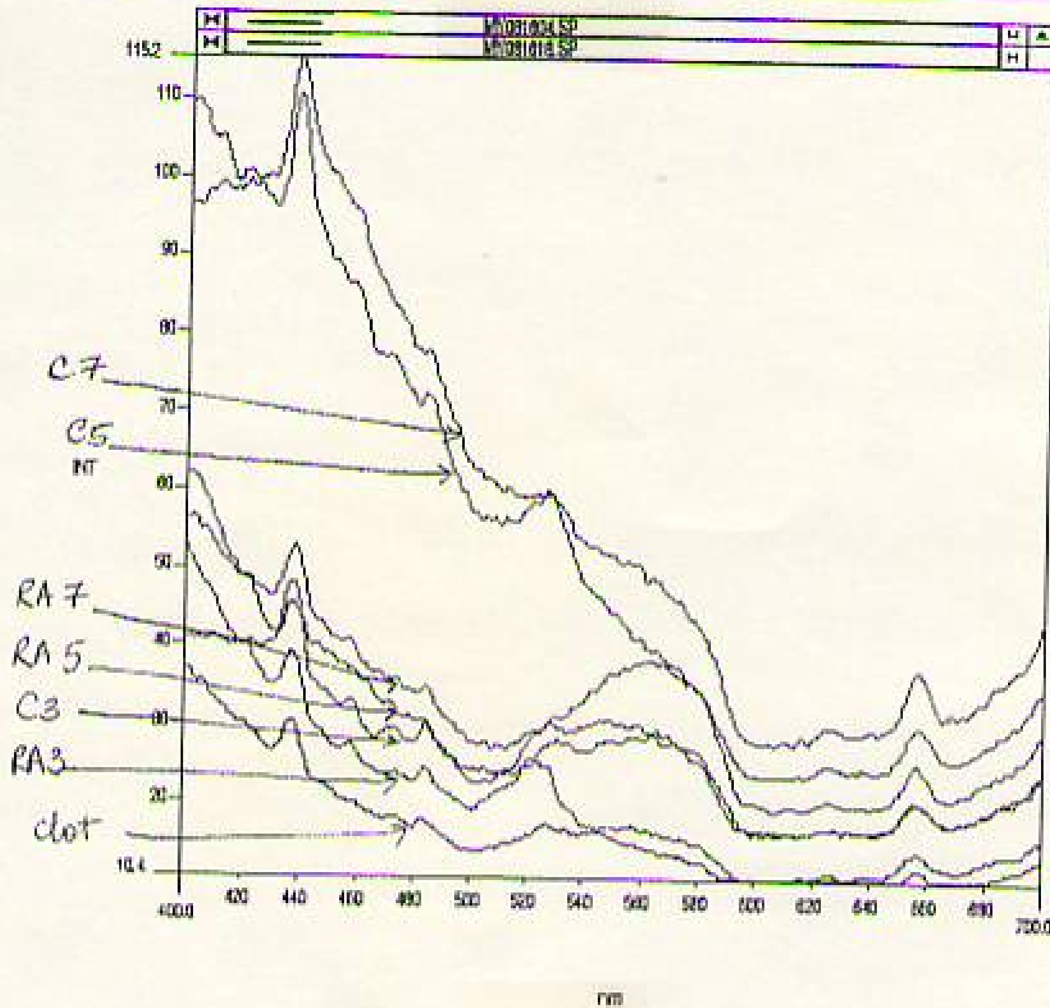
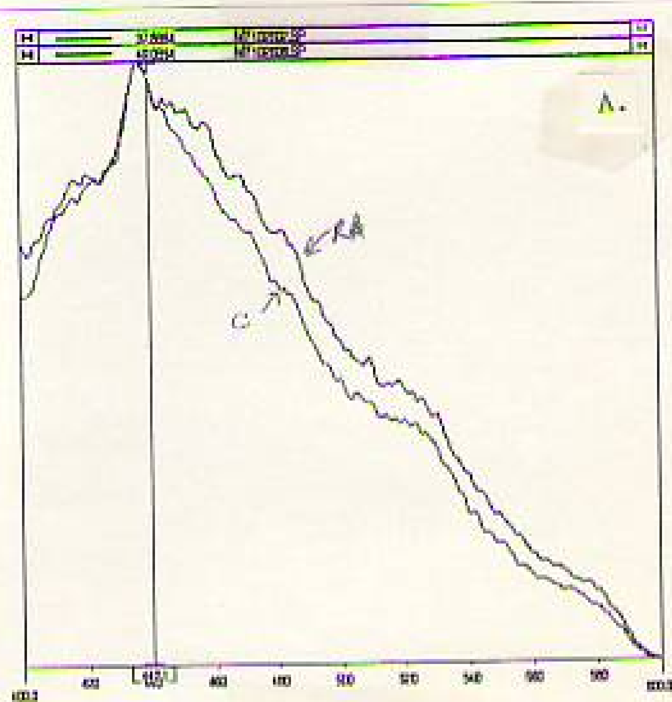
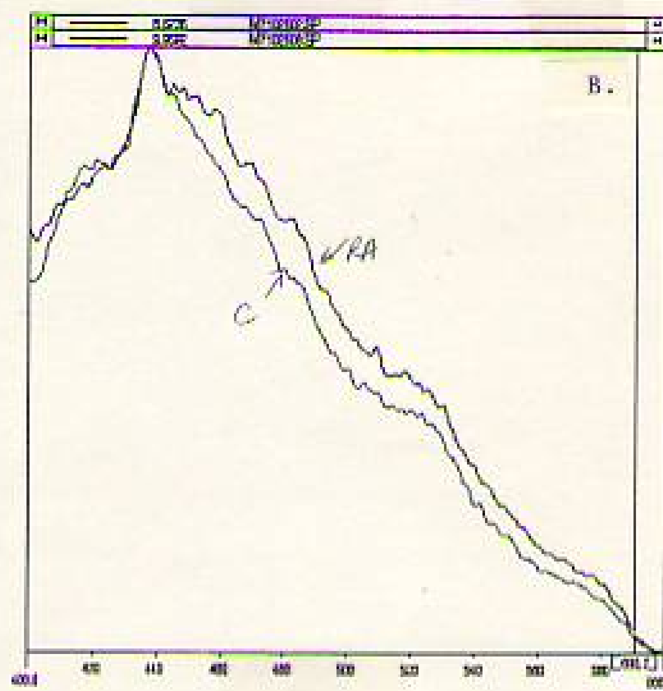


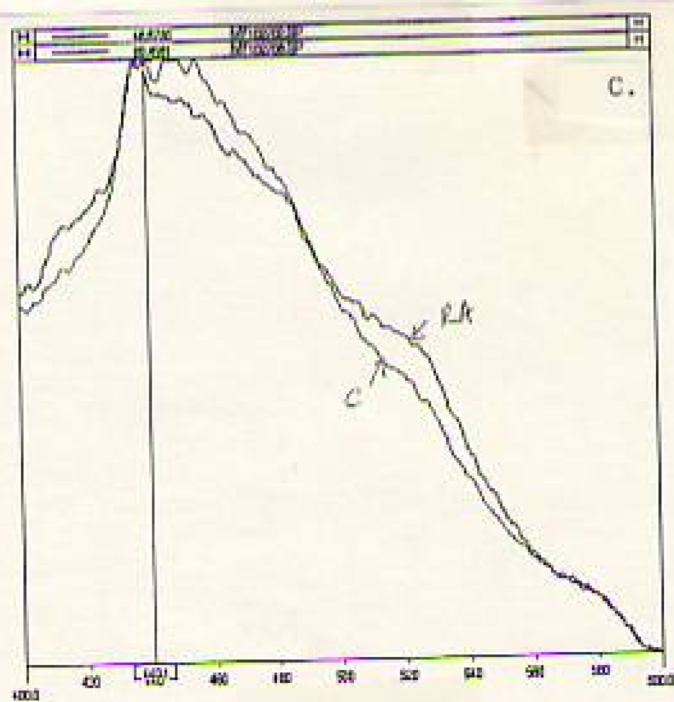
FIGURE 4. SPECTRAL PATTERN OF FIBRIN CLOT AT EMISSION SCAN EX365.

This is a representative scan demonstrating that the spectral pattern emitted by the fibrin clot did not interfere with that of the experimental or control groups. This is an emission scan over the wavelengths 400-700nm, after excitation at 365nm. The intensity is measured in (c.u.) along the y-axis. The control and RA-treated groups are labelled (C or RA) The number following represents the number of days of treatment prior to evaluation.

Figure 5







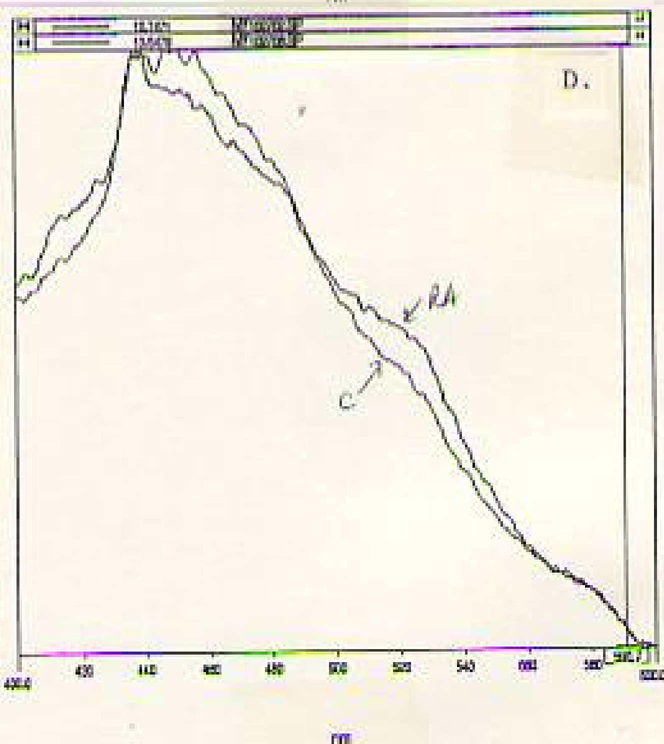


FIGURE 5.

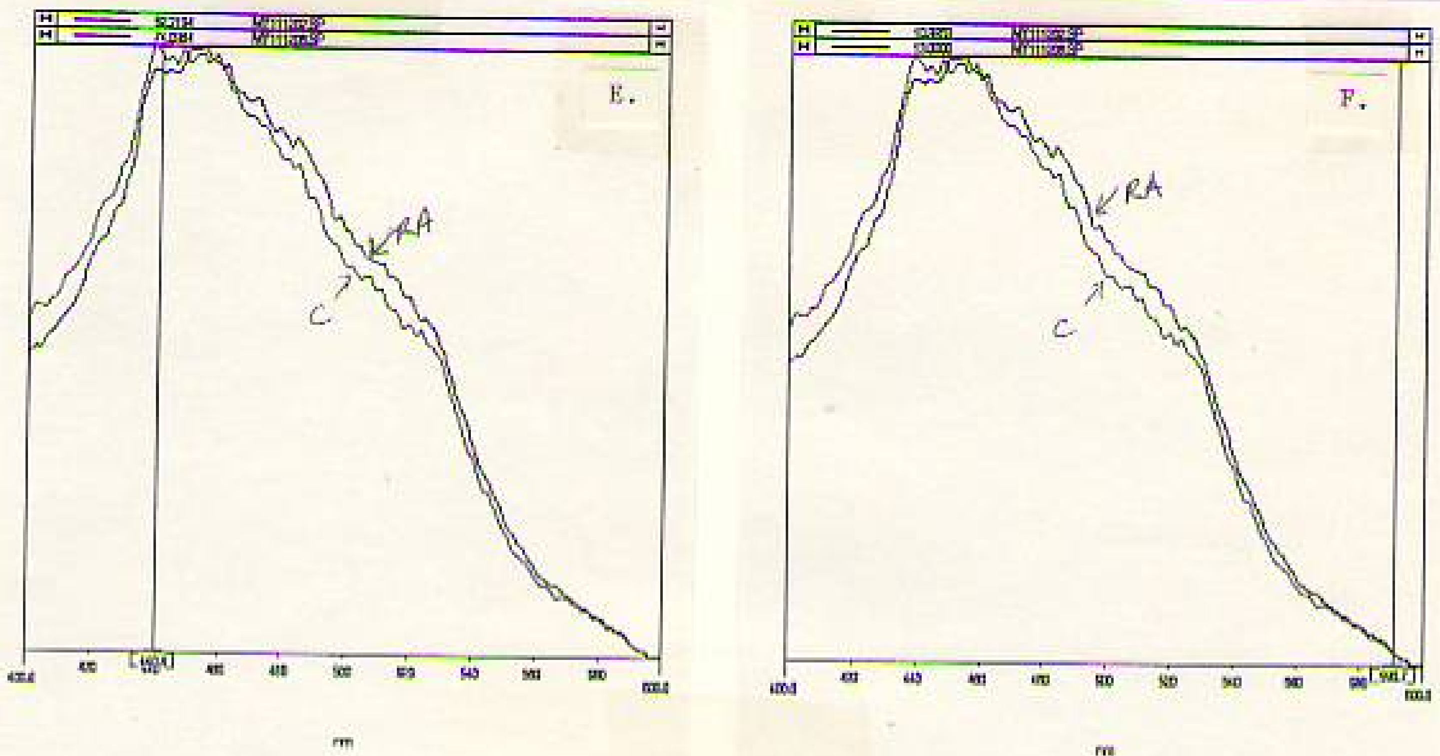
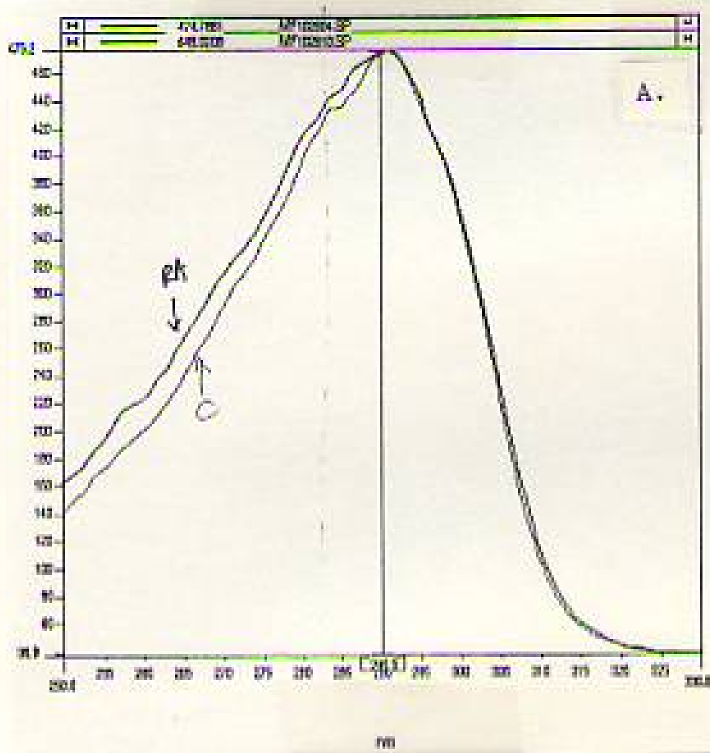
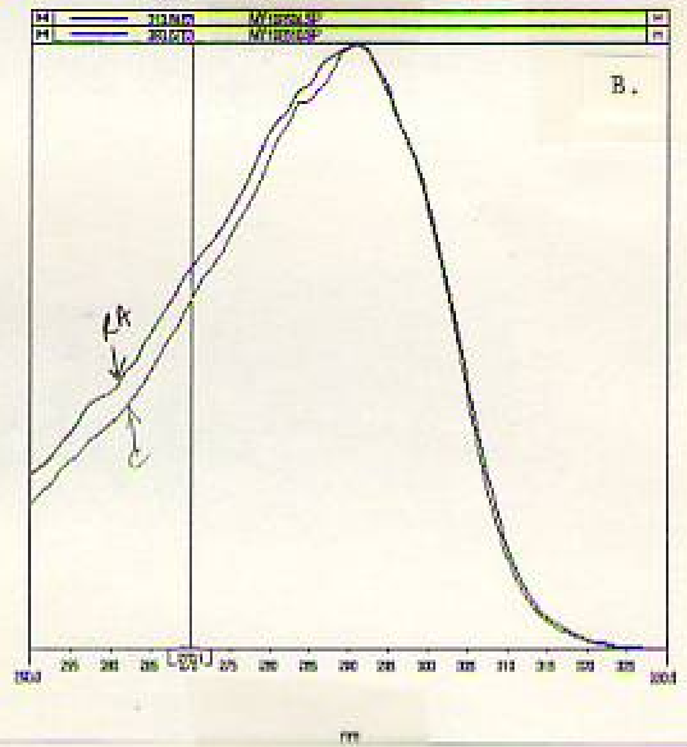


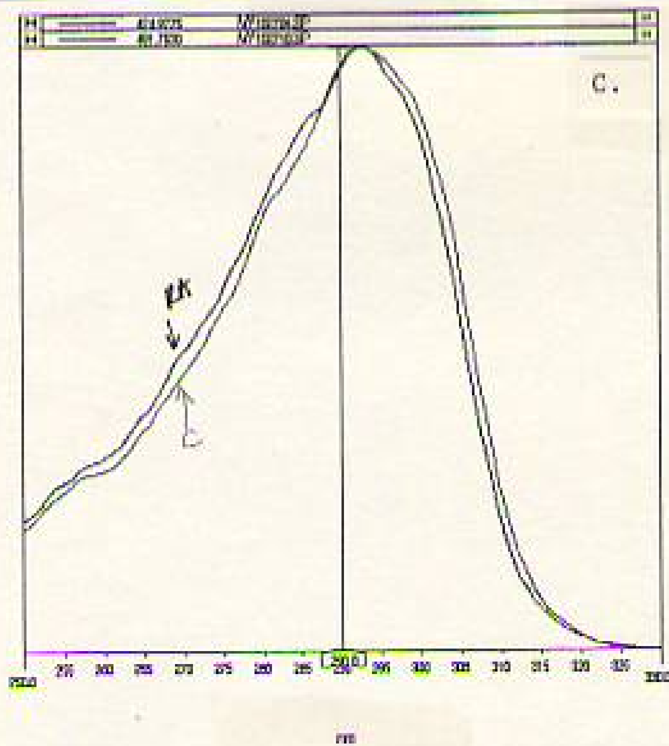
FIGURE 5. EMISSION EX365 (λ 400-700nm)

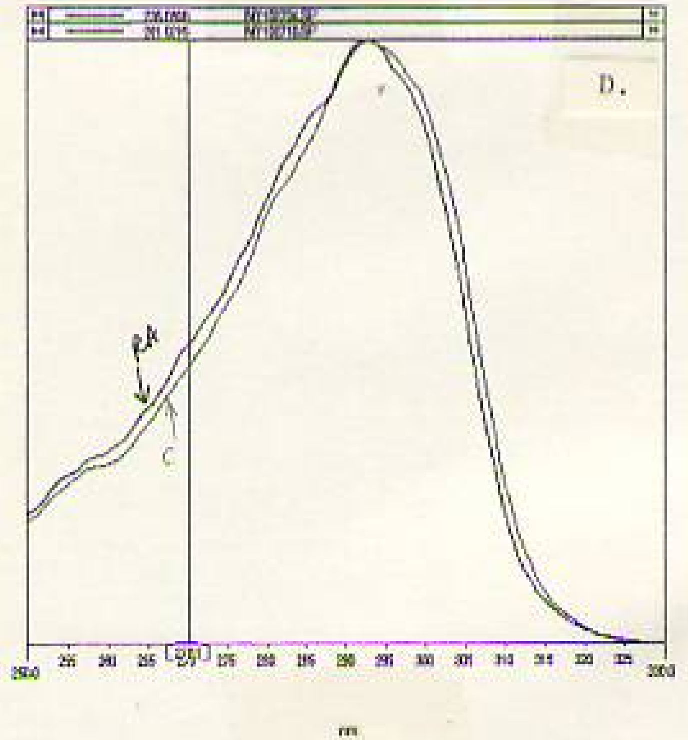
A. Experiment 1. Intensities measured at the 440 nm peak
B. Experiment 1. Intensities measured at the 590 nm peak
C. Experiment 2. Intensities measured at the 400 nm peak
D. Experiment 2. Intensities measured at the 590 nm peak
E. Experiment 3. Intensities measured at the 440 nm peak
F. Experiment 3. Intensities measured at the 590 nm peak
X-axis shows wavelength in nm
Y-axis shows intensity in (c.u.)
RA = 13-cis RA treated group
C = control group
Seven days of treatment

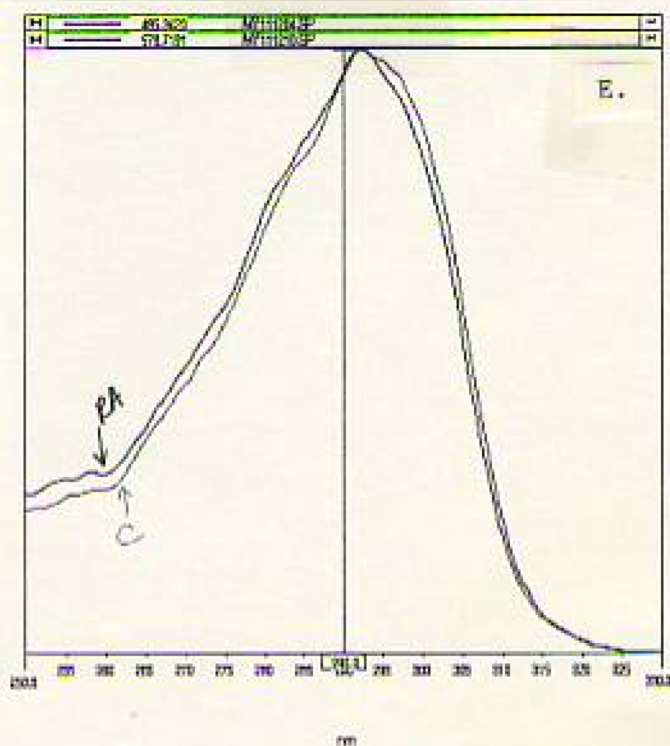
Figure 6











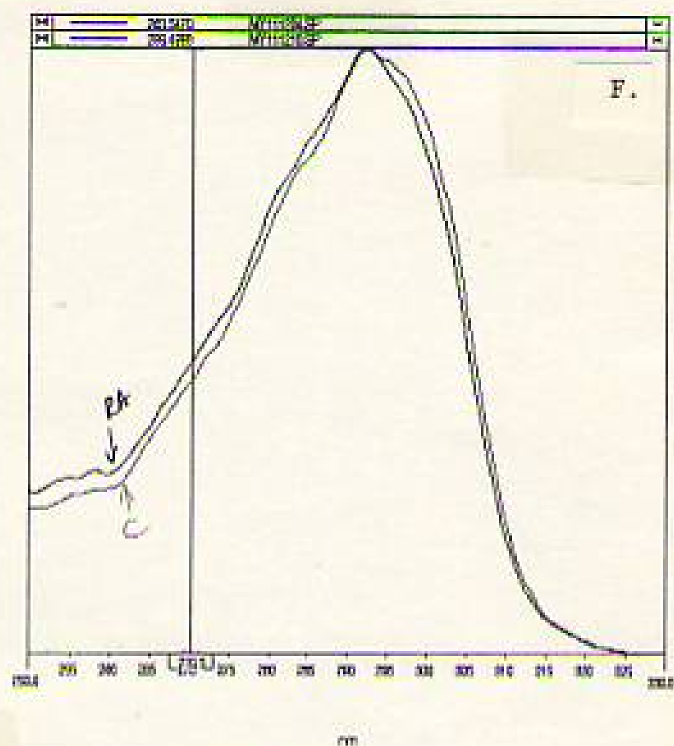


FIGURE 6. EXCITATION EM380 (λ 220-360nm)

A. Experiment 1. Intensities measured at the 290 nm peak
B. Experiment 1. Intensities measured at the 270 nm peak
C. Experiment 2. Intensities measured at the 290 nm peak
D. Experiment 2. Intensities measured at the 270 nm peak
E. Experiment 3. Intensities measured at the 290 nm peak
F. Experiment 3. Intensities measured at the 270 nm peak
X-axis shows wavelength in nm
Y-axis shows intensity in (c.u.)
RA = 13-cis RA treated group
C = control group
Seven days of treatment

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TABLE III

SCAN	EXP #	λRA440nm	λRA590nm	λC440nm	λC590nm	λC-RA440[1]	λC-RA590[2]
EX365	1	38	9	49	10	11	1
	2	49	11	69	13	20	2
	3	62	11	74	13	12	2

SCAN	EXP #	λRA270nm	λRA290nm	λC270nm	λC290nm	λC290/270	λRA290/270	[RA290/270]/[C290/270][3]
EM380	1	313	474	393	646	1.64	1.51	0.13
	2	236	425	259	492	1.90	1.80	0.10
	3	265	487	301	578	1.92	1.84	0.08

*All data is for day seven.

[1] mean=14.33; standard deviation=4.93; standard error of the mean=2.85; t=5.03; $p<0.037$

[2] mean=1.67; standard deviation=0.58; standard error of the mean=0.33; t=5.00; $p<0.038$

[3] mean=0.10; standard deviation=0.03; standard error of the mean=0.02; t=7.11; $p<0.019$