A Determination of the Effectiveness of Macrophages From Sinclair Swine Melanoma on Tumor Cell Cytolysis

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ABSTRACT

The Sinclair Swine Malignant Melanoma is an interesting model for the study of cancer. This melanoma is a spontaneously initiated and regressing tumor. Macrophages have been shown to be infiltrating the tumor at the time of regression during its first stage. The role of the macrophages in regression is not precisely known. The goal of this project was to determine the role of the macrophages on tumor regression. The cytotoxicity of the monocytes from SSCM pigs was evaluated. It was necessary to develop a test system for swine macrophages. The initial assay utilized ^{51}Chromium to label target cells. The current method utilizes a recently developed enzyme-linked colorimetric assay, Cytotox 96®, which measures the activity of lactate dehydrogenase (LDH), an endogenous stable cytosolic enzyme. Four cell lines of mouse mastocytoma cells, P815, P815s, P815K, and P815Ks, were cultured and tested to determine which gave optimum performance in the assay system. The preliminary findings shown that the P815K cell line yields the best results.

BACKGROUND

Cancer is a large problem facing America today, because it is a major cause of death in the United States. Although much information has been learned about cancer over the past few years, there are still a large number of unanswered questions. Cancer is, in a very simple explanation, an uncontrolled growth of cells. Although cell growth is a normal event, cancer arises when the cells begin to multiply out of control, unrestrained by the normal constraints on cell growth. A single abnormal cell may multiply forming a neoplasm, a "relentlessly growing mass of abnormal cells" (Alberts).

A tumor may be described as either benign or malignant. A benign tumor, such as a wart, is a growth of cell that remains encapsulated, usually by connective tissue (Voet and Voet). Benign tumors are rarely fatal. On the other hand, malignant tumors are far more dangerous. They are capable of metastasis, a process by which the tumor cells spread to other parts of the body and develop into secondary tumors. These secondary tumors are usually more serious and often are the cause of death, especially when the metastatic growth occurs in a vital organ and disrupts the normal function of that tissue.

For a tumor to undergo metastasis, it must undergo five major steps: invasion of the surrounding tissue with penetration into lymphatic or vascular channels, release of the neoplastic cell into the circulating blood, survival throughout circulation, arrest in and attachment to a capillary bed, and movement through the capillary wall where the new growth begins (Franks and Teich).

The Sinclair miniature swine cutaneous malignant melanoma (SSCM) is a unique model for the study of cancer. SSCM is inherited and the loci and expression of these genes are currently under investigation. The expression of SSCM has been linked to the Major Histocompatibility Complex (MHC), a region of chromosome 7 in swine (Tissot). Unlike most tumors which occur late in life, the majority of SSCM initiation begins prior to birth at approximately 10 - 14 weeks of gestation. In addition, SSCM normally regresses shortly after birth usually within 6 -12 weeks (Amoss).

The appearance of the tumor first occurs as a nevus, a darken patch of the skin, in the fetal pig. As the cancer progresses, the tumor becomes a solid, darken growth of the skin, similar in appearance to a wart. The tumor is then transformed and the malignant cells either metastasize, resulting ultimately in death of the host, or regress leaving a perfectly healthy pig (Amoss). The regression is now thought to occur in two phases. The first phase occurs around 30-45 days and is thought to be

linked to the infiltration of macrophages. The second occurs after 60 days, usually around 90-120 days is associated with lymphocytes (Green and Amoss, unpublished results).

The immune system is composed of many cell types that are derived from the bone marrow cells. The bone marrow contains pluripotent hemopoietic stem cells (PHSC) which can differentiate into erythrocytes, red blood cells, as well as the leukocytes, white blood cells (Lemischka). Thus, the bone marrow is a mixture of the stem cells, committed progenitor cells, and immature precursor cells (Leenen and Campbell).

The stem cells undergo a variety of changes and replication during development. Proliferation is the replication of a cell resulting in daughter cells with similar characteristics. Maturation is a process in which cell loose features of early development and gain those of later development. Differentiation, although sometimes considered to be synonymous with maturation, is a special case of maturation which generates diversity (Leenen and Campbell). Activation is the process in which cell functions are either changed are enhanced. Of the above processes, activation is the only one which is reversible.

All of the above processes are controlled by a complex system of cell and molecular mediators. These mediators are a vast array of lymphokines including interluekins, interferons and other molecules such as hormones and polysaccharides (Klein).

Macrophages are an important player in the mammalian immune system. They are the first line of defense and the first phagocytic cell to arrive at the invading foreign cell. Macrophages originate from myeloid precursors in the bone marrow. They enter the blood stream as monocytes, an unactivated phagocytic cell with little phagocytic ability. In the blood they comprise 3-8 % of the leukocytes at a concentration of 300 cells per milliliter (Klein). Morphologically, they have a large indented nucleus, many mitochondria, an abundant endoplasmic reticulum, and many dense granules (Schwartz).

Upon entering the tissue, monocytes are activated and are differentiated into macrophages. The monocytes are activated by interferons, which are secreted by activated T-lymphocytes. Within 8 - 12 hours, the monocytes swell and develop many lysosomes capable of digestion (Marieb). Monocytes can also be activated into macrophages by nanogram amounts of lipopolysaccharide, often from bacteria cells. It is for this reason that care must be taken during all preparation to avoid lipopolysaccharide contamination (Haslett). Macrophages can be assigned more specific names based upon the tissue in which they reside. For example, macrophages residing in connective tissue are known as histiocytes, while those in the liver are Kupffer

cells (Klein).

Macrophages exist in heterogenous forms. This heterogeneity is due to several factors: 1) the cells are at different stages in their maturation, 2) the cells are activated by different stimuli, 3) the cells have different lineages (i.e. they are from different precursors), and 4) the cells may be part of a separate population of macrophages that is maintain by local proliferation as opposed to proliferation in the bone marrow (Leenen and Campbell).

The primary role of the macrophage is to engulf foreign particles and present antigens upon their surface as a signal to other immune system cells. The macrophage also serves as a means of cell death through the secretion of tumor necrosis factors, TNF-α and TNF-β. Another function is to secrete Interleukin-1 which activates T-lymphocytes (Klein).

Not only is the immune system important in protecting the body from invading bacteria and viruses, but it is also vital in protecting the body from itself. Paul Erlich originated this idea, which was later restated by Thomas and Burnet (Klein). This theory of immune surveillance against cancer stated that abnormal cells and tumors arose frequently during an animals lifetime but the vast majority were eliminated before the tumors were apparent (Frank and Teich).

Macrophages are shown to be a powerful element of the immune system to several forms of neoplasias. Macrophages, when fully activated, can distinguish between normal and neoplastic cells (Heppner). In order for cytolysis to occur, tumor associated macrophages (TAM) must remain in contact with the tumors cells for a specified length of time, at a certain macrophage density, and at a specific macrophage to tumor cell ratio. These parameters vary between different types of cancer but are reproducible for a specific type of tumor (Heppner).

Cytolysis can occur through numerous processes, one of which is through the secretory products, tumor necrosis factors (TNF). These factors are either cytostatic or cytotoxic to a large array of cells (Marieb). This cytotoxic activity of macrophages occurs only after a priming event, a triggering event such as stimulation by INF-γ and then bacterial lipopolysaccharide (Leenen and Campbell). Inflammatory macrophages incubated overnight with rINF-γ have been shown to have an increased tumorcidal activity (Campbell).

Project

This project began last fall with the perfection of the procedure to isolate monocytes from whole pig blood. The pigs were bled through the retino-orbital plexus, a capillary bed behind the eye (Huhn). The blood sample was then separated on a Histopaque® ficoll gradient. The resulting buffy layer of cells at the interface contained a mixture of all the white blood cells (WBC) from the blood, including blood monocytes. After removing and washing the WBC's, a method of isolating the monocytes was needed. A very simple and commonly used approach is to layer the WBC's onto tissue culture dishes. Because of the adherent nature of the monocytes, they will adhere and spread out on the plate bottom. The other WBC's can then be washed away leaving only a monolayer of monocytes in the tissue culture dish. Several time intervals were tested to determine which gave the optimal number of monocytes attachment within the smallest amount of time. This time length was found to be 1 hour. For the experiment, each well of a 96 well plate was seeded with approximately $2x10^5$ monocytes and left to settle for 1 hr at 37^{o} C. Non-adherent cells were removed by the following washes with fresh media.

The next step of the project was to begin culturing the cell line for the target cells. The ATCC P815 mouse mastocytoma cell lines that had previously been used in the lab were the cells of choice. The lab had two "lines" of these cells: P815, the line obtained from ATCC; and P815K, the same cell line obtained from another lab in Kentucky. Troubles arose with contamination in both of these cell lines when the stocks were thawed and placed into culture. After some time, these cells were successfully cultured in RPMI 1640 media (Gibco) with 10% Fetal Bovine Serum (FBS). The mastocytoma cells are supposed to be an adherent cell line but the cultures had numerous suspension cells that were viable. From these suspension cells, two more cell lines, P815s and P815Ks were subcultured. From these four cell lines, the most effective cell line for the cytotoxicity assay was tested.

The initial assay utilized a standard ^{51}Chromium assay that was originally designed for the P815 cells (Russell). The basis of the assay is the leakage of ^{51}Cr from ruptured or damaged plasma membranes of the target cells, which have been previously labelled with sterile $Na_2{}^{51}CrO_4$. The procedure calls for the addition of labelled target cells (P815) to the effector cells (monocytes) followed by an incubation for 16-20 hours at 37°C. The supernatant of the target and effector cell mixture is then removed and quantified in a gamma spectrometer. The amount of radioactivity is proportional to the number of target cells lysed - upon lysis or other damage to the cell membrane all of the cellular contents, including the radioactive chromium, are released to the media.

The target cells have not been successfully labelled with $^{51}CrO_4$. Two different protocols for labeling have been tried, neither which has worked. The basic outline of the two methods is shown below. The detailed protocols are given in Appendix A.

Old	New
Use 10^5 cells	Use $\geq 10^6$ cells
Use 250 μCi $^{51}CrO_4$	Use 100 μCi $^{51}CrO_4$
Incubate with 1 ml media	Incubate with only Cr
Incubate 37^o for 1 hr	Incubate 37^o for 1.5 hr
Wash & incubate 1 hr (1 ml)	Wash 3X
Wash	

Several people were contacted to obtain the new protocol. Many reasons were discussed for the failure of the old method. These included too much radioactivity which resulted in killing of the target cells, too small of a cell population being labelled, and too dilute of a solution of ^{51}Cr for effective labelling (Bennett). These problems should have been rectified in the new protocol but this was not the case.

As shown by Figure 1, the average spontaneous release for each of the four cell types, P815, P815s, P815K, and P815Ks, was larger than the maximum release. The actual counts are listed in Appendix B in Figures 1-4. These same results were found on the other trials of the ^{51}Chromium cytotoxicity assay. The cause of this anomaly is still unknown.

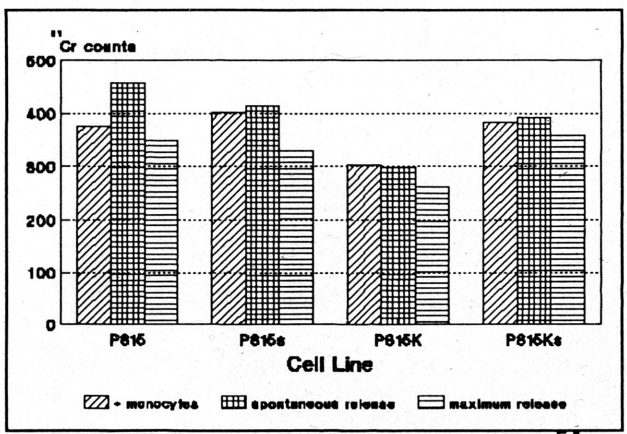

Figure 1 Results from ^{51}Cr labelling experiment of mouse mastocytoma cells.

A new approach is currently being used. It utilizes an enzyme-linked colorimetric assay, Cytotox 96®, which measures the activity of lactate dehydrogenase (LDH), an endogenous stable, cytosolic enzyme that is released upon cell lysis. After target and effector cells are incubated for several hours, the mixture is incubated with tetrazolium salt (INT) for 30 minutes to form formazan, a red product. The amount of formazan product is measured through its absorbance at 490 nanometers. Again, the amount of product, and hence the intensity of color, is proportional to number of cells lysed as with radioactivity assay. The reactions for the assay are outlined below.

$$NAD^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + NADH$$

$$NADH + INT \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan}$$

The initial runs of the Cytotox 96® are to optimize the number of target cells needed in the experimental assays. The optimal range of absorbance is two to three times that of the background absorbance. The protocol given with the kit suggested using 5,000, 10,000, and 20,000 cells per well as a staring point.

The results from the first run of the Cytotox 96® Assay are shown in Figure 2. The actual absorbances are listed in Tables 5 and 6 of Appendix B. A decrease in absorbance was seen with an increase in cell number, as opposed to the expected increased absorbance associated with more cell death. There are several reasons for this discrepancy. The phenol red indicator in the RPMI is reported to cause interferences with the reading at 490 nm. Another contaminant causing an incorrect reading is fetal bovine serum (Korzeniewski). Both of these interferences were then eliminated by using serum free RPMI media without phenol red on all subsequent trials.

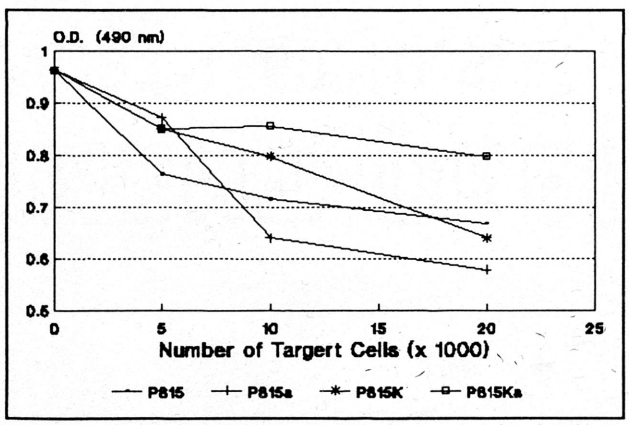

Figure 2 Results from initial Cytotox 96® Assay

In the next run of the Cytotox 96® on March 2, the phenol red indicator in the RPMI and the fetal bovine serum were eliminated. The results were much better than in the previous trial, although there was not as dramatic of an increase in absorbance with the increase in cell numbers as expected. Of the three cell lines tested, P815K showed the most rapid increase in absorbance as shown in Figure 3. The data for this graph is given in Tables 7 and 8 of Appendix B.

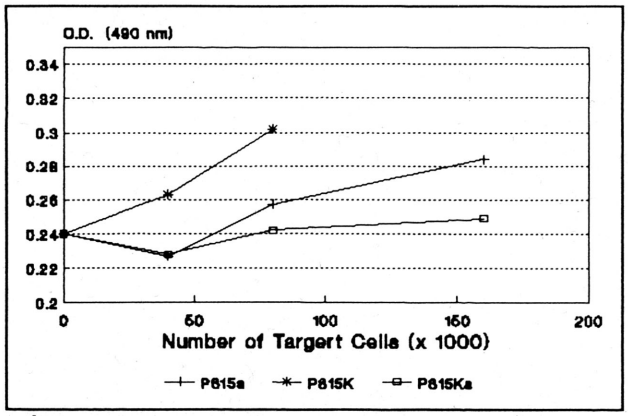

Figure 3 Results from Cytotox96® Assay using phenol red free RPMI and no Fetal Calf Serum.

The experiment was repeated on March 23, but received very different results from the first time. As shown in Figure 4, the only cell line that worked properly in this trial was P815s. This was inconsistent with the previous experiment. The data for this graph is in Tables 9 and 10 of Appendix B.

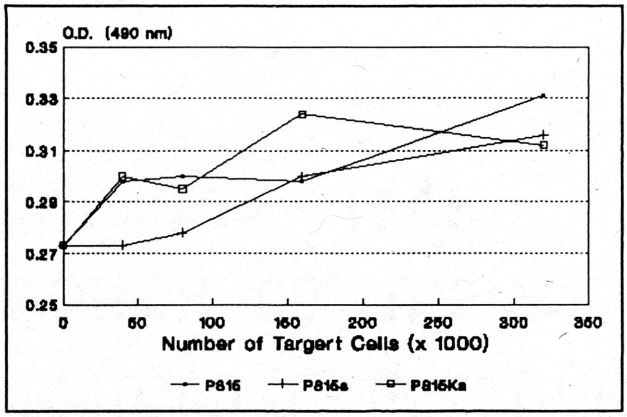

Figure 4 Results from Cytotox 96® Assay

During the course of the experiments, the lab switched to a newer lot of fetal bovine serum. After this change, the P815s cell line proliferated very rapidly while the other three lines remained at their original growth rates. The P815s cell line was tested alone in a wider spectrum of cell numbers (up to 2 million per well); however, the results of the experiment were no better with a larger number of cells. The data is given in Appendix B in Table 11.

The next hypothesis was that the lack of a dramatic response was due to an incomplete lysis of the cell membrane by the lysis solution contained in the Cytotox 96® kit. The was an alternate method given for a freeze-thaw lysis. This lysis was attempted to ascertain if the poor results were indeed due to incomplete lysis. The results did not improve, in fact, they were worse as shown in Appendix B in Tables 12 and 13.

A positive LDH control was included in the Cytotox 96® kit as a method to check the other components of the system. When the positive control was used, the result was a dramatic color change to a deep maroon color. The absorbances were so high that the microplate reader could not read them. The absorbances of the solutions in all the positive control wells were off the scale.

The next step is to try different cell lines to see if another cell lines gives better results. One possibility may lie in the excessive growth of the cells with the change in the fetal bovine serum. This may have changed some aspect of the cell line. Another direction is to re-culture the cells from stock to avoid any changes that might have occurred.

CONCLUSION

It was not possible to address the question of the role of the peripheral macrophages on tumor regression. It still remains to develop the assay system into a routine procedure in this laboratory. Work will continue on the development of the assay itself. Because of the importance of the original question, a significant commitment will be made on this project.

APPENDIX A: PROTOCOLS

I. Monocyte isolation

1. Draw 15 ml blood into a heparinized vacutainer tube.

2. Perform Histopaque separation.
   a. Dilute 5 ml blood in 5 ml PBS.
   b. Add 4 ml room temperature Histopaque to a 15 ml tube.
   c. Carefully overlay the 10 ml of diluted blood.
   d. Spin at 400X g for 45 minutes at room temperature.
   e. Remove and discard upper layer within 0.5 cm of interface.
   f. Transfer the white blood cells at interface to 10 ml of PBS (combine 2 interfaces to 1 tube PBS).
   g. Spin 250 g for 10 minutes.
   h. Remove supernatant, and resuspend pellet in 5 ml PBS.
   i. Spin 250 g for 10 minutes.
   j. Combine all cell portions and resuspend in 1.0 ml PBS.

3. Using cytometer, determine concentrations of WBC.

4. Using 5 % monocyte concentration, determine concentration od monocytes in WBC.

5. Seed 1.5-2.5 x 10^5 monocyte per each well in a 96 well plate, using RPMI media with 10% FBS and 1% AB/AM.

6. Allow cells to adhere for 1 hour while incubating at 37°C.

7. Wash monolayer with fresh media 3 times.

8. Remove all media and replace with .1 ml media.

9. Incubate overnight at 37°C.

II. Chromium Labelling Procedure

A. Original Protocol

1. Use target cells that are in exponential phase of growth.

2. Centrifuge at 200 g for 5 minutes.

3. Resuspend in 0.5 ml RPMI + 10% FBS.

4. Add 250μCi of ^{51}Cr. Bring final volume to 1 ml with RPMI + 10% FBS. (^{51}Cr should not be more than 1 month old).

5. Incubate the solution in 37°C water bath for 1.5 hour with gentle shaking every 10-15 minutes.

6. Terminate the uptake by diluting the solution up to 50 ml with warm RPMI + 10% FBS. Invert gently to mix.

7. Centrifuge at 200 g for 10 minutes to pellet cells.

8. Remove all of the supernatant and resuspend cells in 10 ml warm RPMI + 10% FBS.

9. Incubate in 37°C water bath for 1 hour, gently inverting every 15 minutes.

10. Centrifuge at 200 g for 10 minutes to pellet cells and remove all supernatant.

11. Resuspend cells in a convenient volume and count.

B. Revised Protocol

1. Use target cells that are in exponential phase of growth.

2. Centrifuge at 200 g for 5 minutes.

3. Resuspend in minimal amount of RPMI + 10% FBS.

4. Add 100μCi of ^{51}Cr. (^{51}Cr should not be more than 1 month old).

5. Incubate the solution in 37°C water bath for 1 hour with gentle shaking every 10-15 minutes.

6. Terminate the uptake by diluting the solution up to 50 ml with warm RPMI + 10% FBS. Invert gently to mix.

7. Centrifuge at 200 g for 10 minutes to pellet cells.

8. Remove all of the supernatant and resuspend cells in 10 ml warm RPMI + 10% FBS.

9. Centrifuge at 200 g for 10 minutes to pellet cells and remove all supernatant.

10. Repeat washes (steps 8 & 9) twice.

11. Resuspend cells in a convenient volume and count.

III. Cytotoxicity Assay

1. In 96 well plates, add 0.1 ml target cells to the previously plated monocytes in 0.1 ml RPMI + 10% FBS

2. Incubate at 37°C for 16-18 hours.

3. Spin at 1000 rpms for 5 minutes to pellet cells.

4. Carefully remove 0.1 ml supernatant to a labelled 12mm x 75 mm tube.

5. Quantify radioactivity using a gamma counter.

Controls

1. Spontaneous release - Target cells incubated alone for 16 - 18 hours.

2. Maximum release - 0.1 target cells + 0.1 distilled water. Hold overnight at -20°C, thawed, then freeze-thawed twice the next morning. Tubes are then vortexed, centrifuged at 750 g for 10 minutes. Remove 0.1 ml supernatant to a labelled 12mm x 75 mm tube.

Analysis of Data

Spontaneous Release(SR): radioactivity released from target cells incubated at 37° C alone

Maximum Release(MR): radioactive count from freeze-thaw of target cells

$$\text{Background Release/hr} = \frac{\text{Spontaneous Release}}{(\text{Time})(\text{Maximum release})} \times 100$$

$$\text{Experimental Release} = \frac{\text{Expt - SR}}{\text{MR - SR}} \times 100$$

IV. Cytotox 96® Cytotoxicity Assay

Optimization of Target Cell Number

1. Plate dilutions of cells in 96 well plate in 0.2 ml RPMI + 10% FBS.

2. Prepare media control wells for background absorbance.

3. Add 20 µl lysis solution to all wells.

4. Incubate at 37°C for 45 minutes.

5. Centrifuge plate at 250 g for 5 minutes.

6. Transfer 50 µl of supernatant to new 96 well plate.

7. Reconstitute substrate mix with 12 ml assay buffer and add 50 µl to each well while avoiding direct light.

8. Cover and incubate at room temperature for 30 minutes.

9. Add 50 µL stop solution to all wells.

10. Record absorbance at 490 nm within 1 hour.

APPENDIX B: DATA

^{51}Chromium Labelling Cytotoxicity Assay
December 7, 1992

Table 1: P815 cells results - ^{51}Cr Assay

	P815 + MC	SR	MR
	448	522	359
	368	355	326
	312	395	362
	-	561	-
ave	376	458	349
± σ	68	99	20

Table 2: P815s cells results - ^{51}Cr Assay

	P815s + MC	SR	MR
	455	478	415
	397	361	287
	350	332	282
	-	487	-
ave	401	414	329
± σ	53	79	78

Table 3: P815K cells results - ^{51}Cr Assay

	P815K + MC	SR	MR
	302	381	283
	288	270	251
	320	263	252
	-	282	-
ave	303	299	262
± σ	16	55	18

Table 4: P815Ks cells results - ^{51}Cr Assay

	P815Ks + MC	SR	MR
	373	507	421
	407	385	313
	365	391	344
	-	280	-
ave	382	391	359
± σ	22	93	56

Cytotox 96® Assay
February 23, 1993
OD 490 nm

Table 5: Media background controls (RPMI 1640 + substrate and stop solution) - Cytotox 96®

0.924	0.912	0.912	0.943	0.991	1.002	1.027	0.989

ave ± σ = 0.962 ± 0.045

Table 6: Target cell Results - Cytotox 96®

Cells	5,000	10,000	20,000
P815	0.783	0.729	0.625
	0.778	0.708	0.675
	0.773	0.743	0.698
	0.728	0.688	-
ave	0.765	0.717	0.669
± σ	0.025	0.024	0.032
P815s	0.819	0.642	0.582
	0.907	0.698	0.671
	0.891	0.579	0.480
ave	0.872	0.640	0.578
± σ	0.047	0.059	0.096
P815K	.791	0.755	0.583
	0.869	0.806	0.697
	0.857	0.833	-
	0.887	-	-
ave	0.851	0.798	0.640
± σ	0.042	0.039	0.081
P815Ks	0.883	0.852	0.800
	0.790	0.863	0.765
	0.873	0.881	0.829
	0.855	0.830	-
ave	0.850	0.856	0.798
± σ	0.042	0.021	0.032

Cytotox 96® Assay
March 2, 1993
OD 490 nm

Table 7: Media background controls (RPMI 1640 + substrate and stop solution) - Cytotox 96®

0.222	0.236	0.229	0.219	0.263	0.229	0.271	0.248

ave ± σ = 0.240 ± 0.019

Table 8: Cytotox96® Experimental Results

Cells	40,000	80,000	160,000
P815Ks	0.230	0.226	0.246
	0.227	0.257	0.256
average	0.228	0.242	0.249
P815s	0.225	0.236	0.284
	0.229	0.278	-
average	0.227	0.257	0.284
P815K	0.266	0.307	-
	0.260	0.296	-
average	0.263	0.302	-
P815	0.298	-	-
	0.282	-	-
average	0.290	-	-

Cytotox 96® Assay
March 23, 1993
OD 490 nm

Table 9:Background Controls - Cytotox 96®

RPMI only				RPMI + lysis			
0.265	0.273	0.276	0.276	0.292	0.298	0.320	0.308
0.273 ± 0.005				0.305 ± 0.012			

Table 10: Cytotox 96® Experimental Results

Cells	40,000	80,000	160,000	320,000
P815s	0.272	0.274	0.305	0.317
	0.285	0.282	0.294	0.316
Average	0.276	0.278	0.300	0.316
P815Ks	0.286	0.302	0.329	0.310
	0.315	0.288	0.318	0.324
Average	0.300	0.295	0.324	0.312
P815K	0.298	0.300	0.294	0.332
	0.299	0.300	0.302	0.330
Average	0.298	0.300	0.298	0.331

Table 11: Additional results with extra P815s

640,000		1,280,000				
0.301	0.306	0.313	0.292	0.310	0.322	0.325
0.302	0.307	0.355	0.303	0.326	0.322	0.373
0.304 ± 0.003		0.325 ± 0.024				

Cytotox 96® Assay
March 30, 1993
OD 490 nm

Use of Freeze-thaw lysis on P815s cells.

Table 12: Background Controls - Cytotox 96®

0.139	0.136	0.133	0.132	0.133	0.132	0.143	0.135

Average and Standard Deviation = 0.135 ± 0.004

Table 13: Freeze-thaw lysis results for P815s cells

50	100	200	400	800	1,500		
0.138	0.144	0.145	0.141	0.138	0.147	0.150	0.146
0.142	0.142	0.143	0.132	0.133	0.133	0.134	0.149
0.153	0.135	0.136	0.133	0.163	0.132	0.166	0.149

Average and standard deviation

0.144	0.140	0.141	0.135	0.145	0.137	0.150	0.148
0.008	0.005	0.005	0.005	0.016	0.008	0.016	0.001

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