Wendy M. Bradberry

Tennessee Scholars Project

Leukemia Research at the University of Tennessee Hospital

Project Director: Dr. Ichiki

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ABSTRACT

Information gained in these studies seems to indicate the possibility that the K-562 cell line may partially differentiate to B- cells. It was first shown in previous experiments that anti-hIgM kills K-562 cells. The anti-hIgM also bound to the cells, which indicates that IgM is expressed on the surface of the K-562 cell. Anti-hIgM kills all of the K-562 cells within four hours. A later experiment showed the possibility of patching and capping occurring with anti-hIgM on the surface of the K-562 cell. The antigenic expression of the K-562 cell did not change after treatment with anti-hIgM. To try to save the cell from the anti-hIgM induced death, IgM was added to the cells. The hypothesis was that the anti-hIgM would bind to the IgM, and, therefore, be unable to bind to the cell and cause death. However, the results showed that the IgM was toxic to the cell as well and, when added with anti-hIgM, enhanced the toxicity.

Anti-hIgD was also tested to determine its effect on the K-562 cell. It was toxic to the cell. This offers the possibility of more evidence of the K-562 cell line expressing characteristics of immature B- cells. If the anti-hIgD binds to the cell, it would show that both IgM and IgD are expressed on the cell surface. These data show that (a) Both anti-hIgM and anti-hIgD are toxic to the K-562 cell (b) Anti-hIgM binds to the K-562 cell (c) IgM does not save the cell from anti-hIgM induced death (d) There is evidence supporting the possibility of apoptosis as the mechanism of cell death (e) There is some evidence suggesting patching and capping of anti-hIgM on the surface of the K-562 cell (f) These K-562 cells show some characteristics normally associated with B-cells.

INTRODUCTION

The K-562 cell line is a human cell line isolated from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis (1). K-562 can be caused to undergo partial differentiation into erythrocytic, granulocytic, lymphocytic, monocytic, and megakaryocytic lineages by several inducers (2). However, K-562 has never been shown to partially differentiate into a B- cell. These studies seem to indicate this as a possibility. A B- cell has been shown to have IgD and IgM on its surface. Normally, only IgM is expressed in the early stages of differentiation. IgD is only

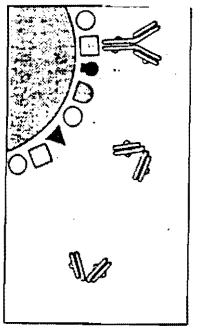
expressed in the later stages, mostly on mature B- cells. Both anti-hIgM and anti-hIgD have been shown to be toxic to the K-562 cell. Anti-IgM does bind to the K-562 cell, but it is not yet known if the anti-hIgD will bind. If the anti-IgD binds, it would seem likely that over the course of many passages, the K-562 cell has acquired certain characteristics normally associtated with B-cells.

MATERIALS AND METHODS

Reagents

The following reagents and media were used: Goat F(ab')$_2$ Anti Human IgM (TAGO, INC. Burlingame, CA); Standard Immunophenotyping Monoclonals (Becton Dickinson Immunocytometry Systems); RPMI 1640 + 10% Fetal Calf Serum; RPMI 1640 + 1% Nutridoma (Boehringer Mannheim Corporation, Indianapolis, IN); hIgM (Sigma Immuno Chemicals, St. Louis, MO); hIgG (Sima Immuno Chemicals, St. Louis, MO); and Goat F(ab')$_2$ Anti Human IgD (TAGO, INC, Burlingame, CA)

This figure shows the difference between the complete antibody and the F(ab')$_2$ fragments used in these experiments.



F(ab')₂ Fragments
Illustrated is a visualization of undesired binding at an F_c receptor. To avoid antibody binding to F_c receptor sites, TAGO provides F(ab')₂ fragments for assays requiring exquisite sensitivity and specificity.

Key:

F(ab')₂

Whole Antibody

F_c Receptor

Cell Preparation

Fresh K-562 cells were obtained from Dr. Lozzio every week to assure that the cell line remained the same throughout the experiments. The cells were used during

the log phase of growth. A concentration of 3.75 x 10^4 cells/ml were used in each experiment.

Determination of the Toxicity of Anti-hIgM

K-562 cells in logarithmic growth phase were adjusted to a concentration of 3.75 x 10^4 cells per milliliter. Two plates were prepared. The first two rows of each plate were cell controls. Fifty microliters of RPMI 1640 + 10% Fetal Calf Serum was added to all wells except the first well of rows C,D,E,F,G, and H of both plates. In well one of these rows, one hundred microliters of : anti-hIgM + azide in rows C,D, and E of plate 1; anti-hIgM - azide in rows F,G, and H of plate 1; anti-CD33 + azide in rows C,D, and E of plate 2; anti-CD33 - azide in rows F,G, and H of plate 2. 100 microliters of K-562 cells were added to all 96 wells. This was incubated at 37 degrees Celsius for 48 hours. 50 microliters of tritiated Thymidine was then added to pre-label the samples. The cells were incubated for three more hours. The cells were then harvested onto glass fiber filter paper and the results were quantitated with a liquid scintillation counter (Beckman Instruments, Inc, Norcross, Georgia.)

Determination of Binding of the K-562 Cell to Anti-hIgM and the Antigenic Profile of the K-562 Cell

The Monoclonal and Leukemia panels shown below were used to determine if anti-hIgM binds to the cell. This same panel was used to determine the antigenic profile of the K-562 cell. The appropriate amount of monoclonal was added to each tube, then .1 ml of K-562 cells were added. The tubes were incubated in the dark at room temperature for fifteen minutes. Two ml of phospate buffered saline (PBS) without calcium and magnesium containing 0.1% Sodium Azide was then added to each tube. They were then vortexed and centrifuged at 250 x g for five minutes. The supernatant fluid was aspirated and .5 ml of 1% paraformaldehyde was added to each tube. They were vortexed. The preparations were stored at four degrees Celcius until they were analyzed on the FACScan (Becton Dickinson Immunocytometry.)

Protocol for two color analysis studies.

Monoclonal Panel

1. Simultest LeucoGATE	(FITC-CD45/PE-CD14)
2. Simultest Control gamma 1/gamma 2a	
3. Simultest CD4/CD8	(FITC-Leu3/PE-Leu2)
4. Simultest CD3/CD19	(FITC-Leu4/PE-Leu12)
5. Simultest CD3/Anti-HLA-DR	(FITC-Leu4/PE-anti-HLA-DR)
6. Simultest CD57/CD8	(FITC-Leu7/PE-Leu2)
7. Simultest CD3/CD16 + CD56	(FITC-Leu4/PE-Leu11c + 19)
8. Combination CD2/CD20	(FITC-Leu5b/ + PE-Leu16)
9. Combination CD2/CD25	(FITC-Leu5b/ + PE-anti-IL-2R)
10. Combination anti-TCR/CD3	(FITC-Leu-anti-TCR/ + PE-Leu 4)
11. Simultest CD3/CD8	(FITC-Leu4/PE-Leu2)
12. Simultest CD3/CD4	(FITC-Leu4/PE-Leu3)
13. Combination anti-hIgm/CD20	(FITC-anti-hIgM/PE-Leu 16)

Leukemia Panel

14. Simultest anti-Kappa/anti-Lambda	(FITC-Kappa/PE-Lambda B-D)
15. Combination CD15/CD33	(FITC-LeuM1 + PE-LeuM9)
16. Combination CD10/Cd20	(FITC-CALLA + PE-Leu16)
17. Combination CD2/CD5	(FITC-Leu5b + PE-Leu1)
18. Combination CD34/CD20	(FITC-CD34 + PE-Leu16)
19. Polyclonal control	Tago (1:10 dilution, 50 microliters)
20. Polyclonal kappa/lambda	Tago (FITC-Kappa + PE-Lambda)
21. Combination CD14/CD33	(FITC-LeuM3 + PE-Leu-M9)

Determination of the Timecourse of anti-hIgM Killing of K-562 Cells

One plate was prepared. All rows contained in wells 1-6 100 microliters of media and 50 microliters of K-562 cells at a concentration of 3.75×10^4. In wells 7-12 there was 100 microliters of anti-hIgM and 50 microliters of cells. The plate was then

incubated at 37 degrees Celcius. One row was treated with 50 microliters of tritiated thymidine at each of the following time points: 4 hours, 8 hours, 24 hours, 28 hours, 32 hours, and 48 hours. The rows were harvested three hours after the time at which the tritiated thymidine was added. The cells were harvested onto glass fiber filter paper. Radioactivity was quantified with a liquid scintillation counter.

Determination of Anti-hIgM Toxicity in Fetal Calf Serum and Nutridoma

One plate was set up. Row A contained 50 microliters of RPMI 1640 + 10% Fetal Calf Serum (FCS) and 100 microliters of K-562 cells grown in FCS. Row B contained 50 microliters of RPMI 1640 + 1% Nutridoma (N) and 100 microliters of K-562 cells grown in Nutridoma media. Rows C,D, and E contained 50 microliters of FCS in wells 2-12, 100 microliters of anti-hIgM in well 1, 50 microliter serial dilutions were done, and 100 microliters of K-562 cells in FCS were added. Rows F,G, and H contained 50 microliters of N in wells 2-12, 100 microliters of anti-hIgM in well 1, 50 microliter serial dilutions were done and 100 microliters of K-562 cells were added. The plate was incubated at 37 degrees Celsius for 48 hours. The cells were treated with 50 microliters of tritiated Thymidine. Three hours later, they were harvested onto glass fiber filter paper, and then raioactivity was quantified with a liquid scintillation counter.

Determination of the Antigenic Profile Before and After Treatment with Anti-hIgM

The same panel was used that was shown in the experiment to determine if Anti-hIgM bound to the K-562 cell. The same procedure was run on the panel. Two of these panels were done; one containing untreated K-562 cells and one containing cells that had been treated with anti-hIgM.

Determination of the effect of hIgM on anti-hIgM Induced Cell Death

One plate was set up. Row A contained 100 microliters of FCS and 100 microliters of K-562 cells. Row B contained 50 microliters of FCS, 50 microliters of hIgM, and 100

microliters of K-562 cells. Rows C,D, and E contained 50 microliters of FCS in wells 2-12, 100 microliters of hIgM in well 1, 50 microliter serial dilutions were done, 50 more microliters of FCS were added, and 100 microliters of K-562 cells were added. Rows F,G, and H contained 50 microliters of FCS in wells 2-12, 100 microliters of hIgM in well 1, 50 microliter serial dilutions were done, 50 microliters of anti-hIgM was added, and 100 microliters of K-562 cells were added. The plate was incubated at 37 degrees Celcius for 48 hours. The cells were then tritiated and harvested three hours later. Quantitative results were obtained from a liquid scintillation counter.

Determination of the Effect of IgM on anti-hIgM Binding to the K-562 Cell

Three test tubes were set up.

Test Tube 1: 20 microliters anti-CD20
20 microliters anti-hIgM (.89 mg/ml)
20 microliters of IgM (1mg/ml)

Test Tube 2: 20 microliters anti-CD20
20 microliters anti-hIgM
40 microliters IgM

Test Tube 3: 20 microliters anti-CD20
20 microliters anti-hIgM
80 microliters IgM

0.1 ml of K-562 cells were added to each tube. The admixture was incubated at room temperature in the dark for fifteen minutes. They were then washed with 2 ml of phospate buffered saline without calcium and magnesium containing 0.1% sodium azide, vortexed, and centrifuged at 250 x g for 5 minutes. The supernatant fluid was aspirated and .5 ml of 1% paraformaldehyde was added to each tube and vortexed. Preparations were stored at 4 degrees Celsius until analyzed by FACScan.

Determination of the Binding of Anti-hIgM to K-562 Cells at Different Times
Determination of Patching and Capping

Four sets of identical tubes were set up. Each set contained one of each of the following tubes:

1. 20 microliters anti-CD4-FITC
   20 microliters anti-CD8-PE
2. 20 microliters anti-CD20-PE
   20 microliters anti-hIgM-FITC

The first tube in each set serves as a negative control since neither bind to the K-562 cell. K-562 cells were added at different time points so that the percent of cells positive for anti-hIgM binding could be obtained for the following time periods: 15 minutes, 60 minutes, 105 minutes and 165 minutes. The results were obtained by analysis by FACScan.

Determination of the Effect of IgG on K-562 Cells

A single plate was set up. Rows A and B were cell controls which contained 50 microliters of media and 100 microliters of K-562 cells. Rows C,D, and E contained media in wells 2-12 and 100 microliters of IgM in one. 50 microliter serial dilutions were done and 100 microliters of K-562 cells were added. Rows F,G, and H contained 50 microliters of media in wells 2-12 and 100 microliters of anti-hIgG in 1. 50 microliter serial dilutions were done and 100 microliters of K-562 cells were added. 50 microliters of tritiated Thymidine was added after 48 hours of incubation at 37 degrees Celcius. 3 hours later, the cells were harvested onto glass fiber filter paper and analyzed by a liquid scintillation counter.

Determination of the Effect of Anti-hIgD on K-562 Cells

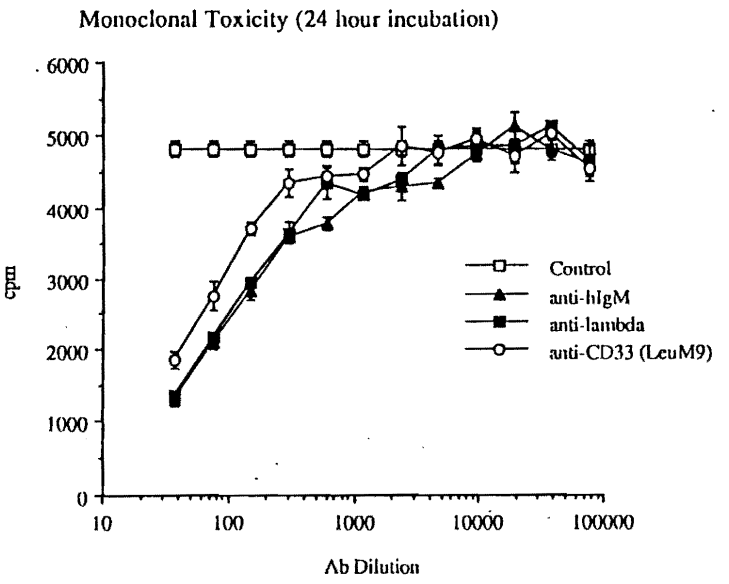
A single assay plate was set up. Rows A and B were cell controls containing 50 microliters of FCS and 100 microliters of K-562 cells. Rows C,D, and E contained 50 microliters of FCS in wells 2-12 and 100 microliters of anti-hIgD + azide in well 1. 50

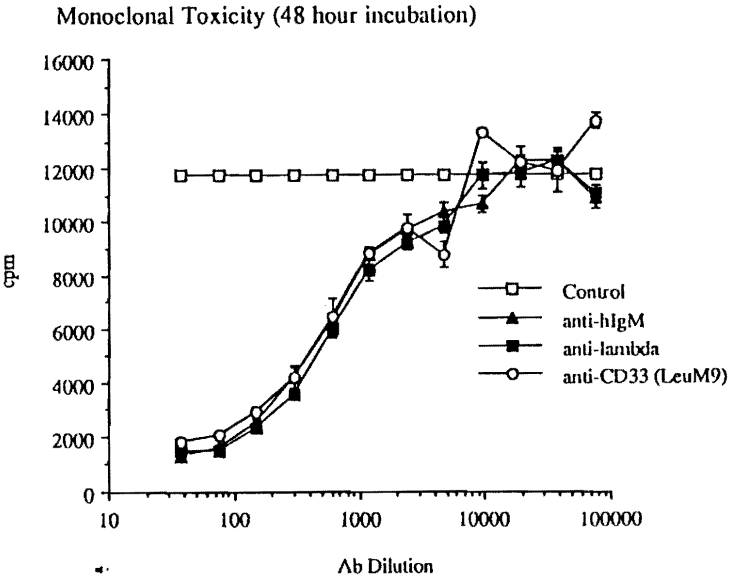
microliter serial dilutions were done and 100 microliters of K-562 cells were added. Rows F, G, and H contained 50 microliters of FCS in wells 2-12 and 100 microliters of anti-hIgD - azide in well 1. 50 microliter serial dilutions were done and 100 microliters of K-562 cells were added. The plate was incubated at 37 degrees Celsius for 48 hours. 50 microliters of tritiated thymidine were added and 3 hours later, the plate was harvested and counted by a liquid scintillation counter.

RESULTS

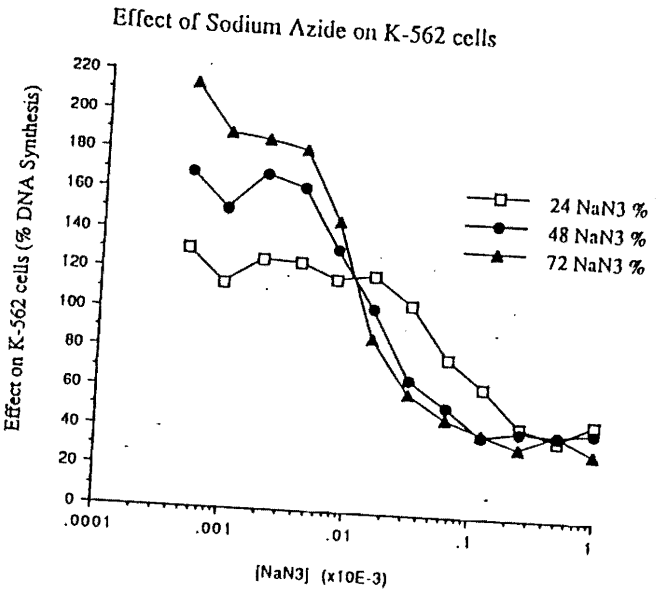
Toxicity of Anti-hIgM on K-562 Cells

Anti-hIgM was shown to be toxic in preliminary experiments done in Dr. Ichiki's lab. These experiments were repeated and the same results were obtained. The following graph shows the results of this initial experiment.

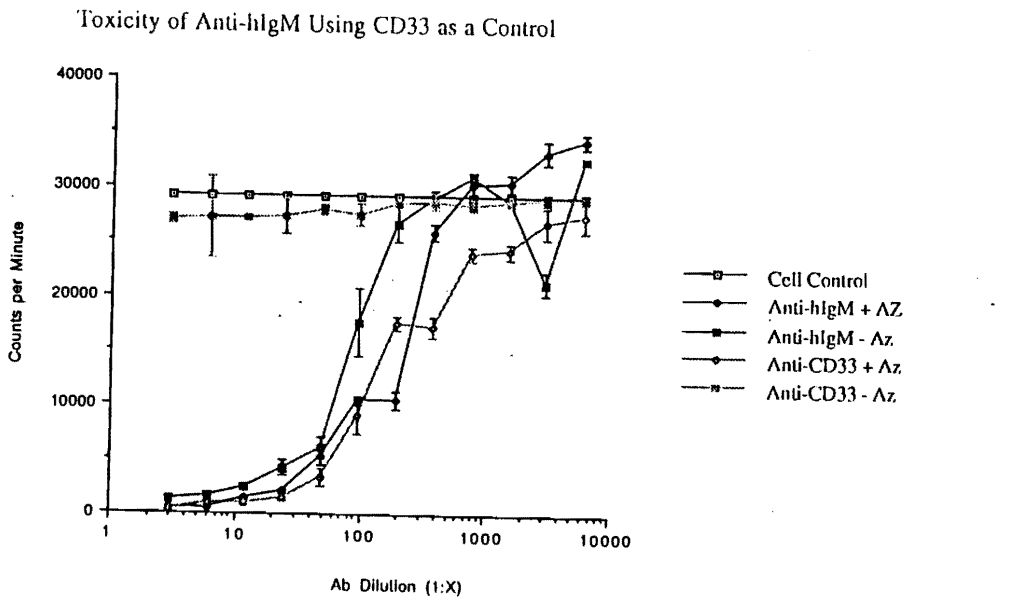




Since the toxicity curves for anti-hIgM, anti-lambda, and anti-CD33 were all exactly the same, there was the possibility of some common factor being the cause of cell death. Upon examination, it was determined that all three contained the same concentration of Sodium Azide. The following graph shows the effect of Sodium Azide on the K-562 cell.



Obviously, the toxicity previously observed was due to the Sodium Azide. Therefore, the next step was to remove the Sodium Azide to see if the anti-hIgM remained toxic to the cell. The results from this experiment confirm that anti-hIgM is toxic to the cell in the absence of Sodium Azide. The first experiment done to show the toxicity of anti-hIgM in the absence of Sodium Azide used anti-CD4 as the control. Anti-CD4 is not toxic to the K-562 cell, but it is also known that it does not bind to the K-562 cell. Since anti-hIgM does bind to the K-562 cell, a better control would be one that did bind, but was not toxic. Therefore, a second toxicity experiment was done using CD33 as a control. It was known that the Sodium Azide was successfully removed, since CD33 was no longer toxic to the cell. The following graph shows the toxicity of anti-hIgM after the removal of Sodium Azide.

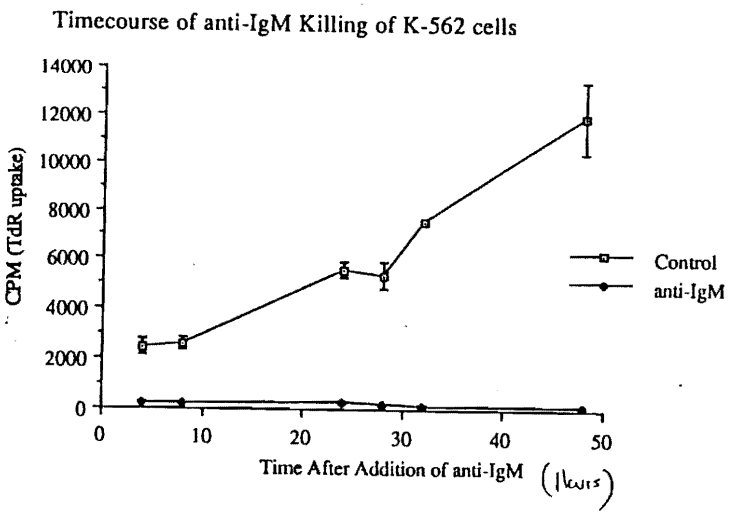


Binding of Anti-hIgM to the K-562 Cell

The K-562 cell was 42% positive for both anti-CD20 and anti-hIgM. It was 2% positive for just anti-hIgM. These results are from only one time point (15 minutes.) The results from a subsequent experiment which is discussed later shows several time points for the binding of anti-hIgM to the K-562 cell. In this experiment, at 15 minutes 80.57% of the cells were positive for anti-CD20 and anti-hIgM. It can be concluded from both of these sets of data that anti-hIgM does bind to the K-562 cell.

Time course of K-562 Cell Death Due to Anti-hIgM

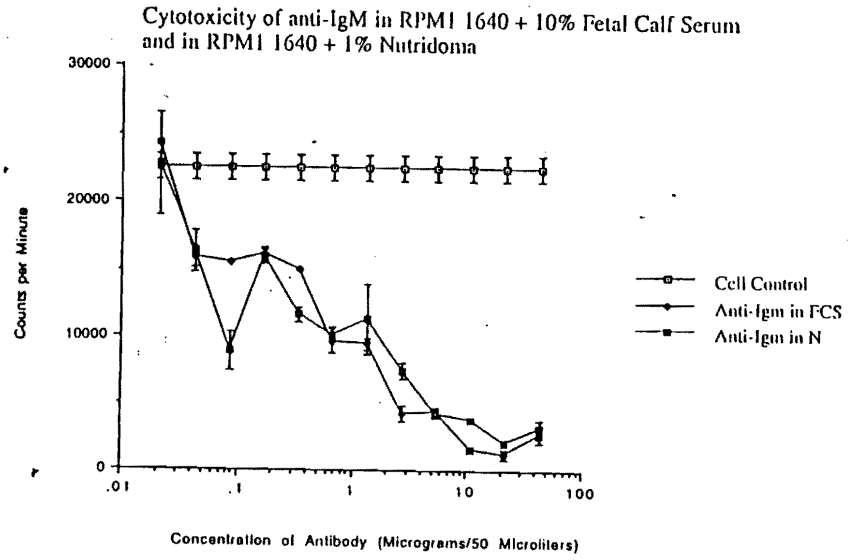
In an assay done to observe K-562 cells treated with anti-hIgM at different time points, it was discovered that the anti-hIgM has induced death by four hours. The following graph shows the time course for cell death.



Several observations were made about the phenotype of the K-562 cell after treatment with anti-hIgM. Pictures 1,2, and 3 are untreated cells and pictures 4,5,and 6 are treated cells. The treated cells show both blebbing and condensation of chromatin. This suggests that the cell death induced by anti-hIgM might be occurring by apoptosis of the cell. Both of these phenotypic characteristics are common in apoptotic cells. Further analysis will have to be done to determine whether or not this is truly the mechanism of cell death.

Toxicity of Anti-hIgM in Fetal Calf Serum Compared to Nutridoma

RPMI 1640 with 1% Nutridoma has the minimal growth factors necessary to sustain life, while RPMI 1640 with 10% Fetal Calf Serum has an excess of growth factors. The hypothesis previous to this experiment was that a lower concentration of anti-hIgM would kill the cells if they were grown in Nutridoma rather than Fetal Calf Serum. If this had proved to be true, it would have been more cost efficient to use the Nutridoma. However, the results show that the toxicity curve is the same for both Fetal Calf Serum and Nutridoma. The following graph shows these results.



Antigenic Profile of the K-562 Cell

The antigenic profile of the K-562 Cell both before and after treatment with anti-hIgM was observed. It did not change any after the treatment with the anti-hIgM. The following tables show the fingerprint of the K-562 cell before and after treatment with

anti-hIgM.

UNIVERSITY OF TENNESSEE MEDICAL CENTER

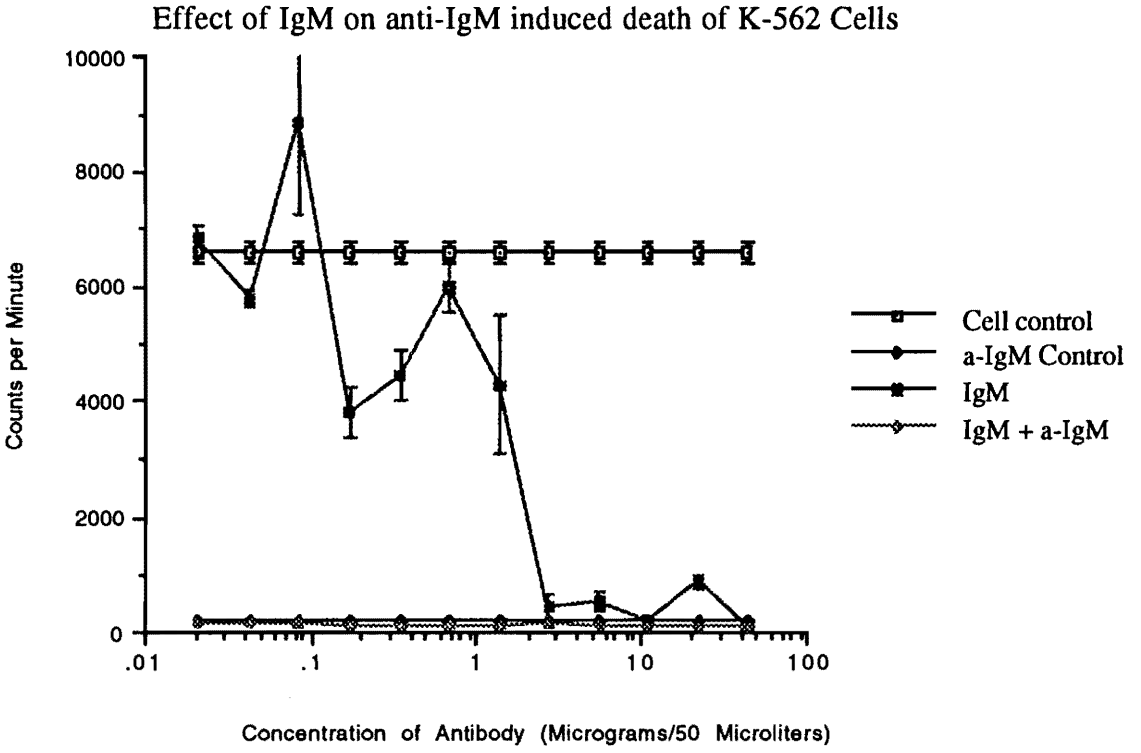
atient ID: K-562'S 4/26/93 Date: 26-Apr-93
Time: 2:39

Tube Name	Cell Type	% L	% L
LEU3/2	CD 8 + CELLS	0	0
	CD 4/ CD 8 + CELLS	0	0
	CD 4 + CELLS	0	0
LEU 4/12	CD 19 + CELLS	0	0
	CD 3/ CD 19 + CELLS	0	0
	CD 3 + CELLS	0	0
LEU 4/HLA-DR	ANTI-HLA-DR + CELLS	0	0
	CD 3/ANTI-HLA-DR + CELLS	0	0
	CD 3 + CELLS	1	1
LEU 7/LEU 2	CD 8 + CELLS	0	0
	CD 57/CD 8 + CELLS	1	1
	CD 57 + CELLS	2	3
LEU 4/11c+19	CD 16/CD 56 + CELLS	0	1
	CD 3+/CD 16+CD 56+ CELLS	0	1
	CD 3 + CELLS	0	0
LEU5B/LEU16	CD 20 + CELLS	84	90
	CD 2/CD 20 + CELLS	1	1
	CD 2 + CELLS	0	0
LEU5B/IL2R	CD 25 + CELLS	21	26
	CD 2/CD 25 + CELLS	1	0
	CD 2 + CELLS	0	0
TCR/LEU 4	TCR/CD 3 + CELLS	1	0
	CD 3+ CELLS	0	0
	TCR+ CD 3- CELLS	2	2
LEU4/LEU2	CD8+ CD3- T-CELLS	0	0
	CD8+ CD3+ T-CELLS	0	0
	CD8- CD3+ T-CELLS	0	0
LEU 4/LEU 3	CD4+ CD3- T-CELLS	0	0
	CD4+ CD3+ T-CELLS	0	0
	CD4- CD3+ T-CELLS	0	0
BD/K+L	BD LAMBDA POSITIVE	0	0
	KAPPA & LAMBDA POSITIVE	0	0
	BD KAPPA POSITIVE	0	0
LEUM1/M9	CD 33 POSITIVE	12	9
	CD 15 & CD 33 POSITIVE	57	63
	CD 15 POSITIVE	25	23
CALLA/LEU16	CD 20 POSITIVE	88	82
	CD 10 & CD 20 POSITIVE	1	0
	CD 10 POSITIVE	0	0
LEU5B/LEU1	CD 5 POSITIVE	0	1
	CD 2 & CD 5 POSITIVE	0	1
	CD 2 POSITIVE-TOTAL T CELLS	0	0
CD 34/LEU 16	CD 20 POSITIVE	89	85
	CD 34 & CD 20 POSITIVE	0	1
	CD 34 POSITIVE	0	0
TAGO/K+L	TAGO LAMBDA POSITIVE CELLS	2	1
	TAGO KAPPA POSITIVE CELLS	1	0
M3/M9	M9,CD 33 POSITIVE	74	66
	M3,M9+: CD 14, CD 33 POSITIVE	1	0
	M3,CD 14 POSITIVE	0	0

Effect of hIgM on Anti-hIgM Induced Cell Death

It was expected that hIgM would bind to anti-hIgM and prevent it from binding and/or killing the cell. The results showed that hIgM did not save the cell from anti-hIgM induced death. It actually killed the cell itself. IgM enhanced the toxicity of anti-hIgM

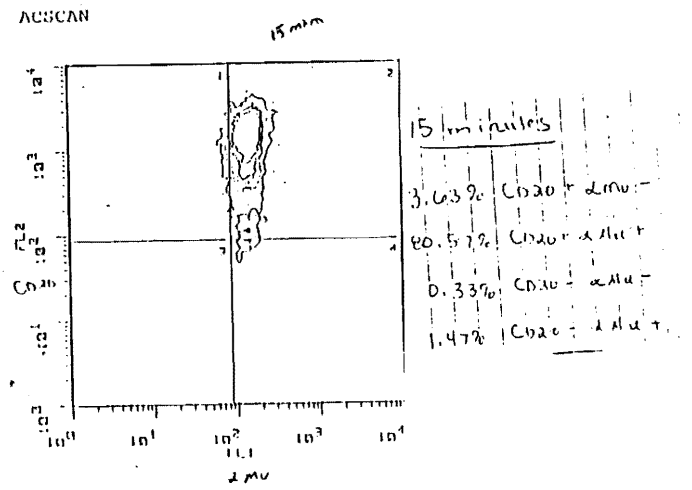
when they were added together. The following graph shows the toxicity of hIgM and the toxicity of anti-hIgM in the presence of hIgM .

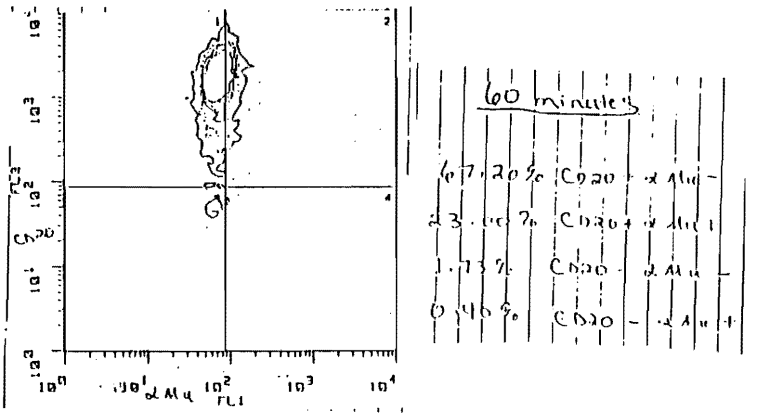


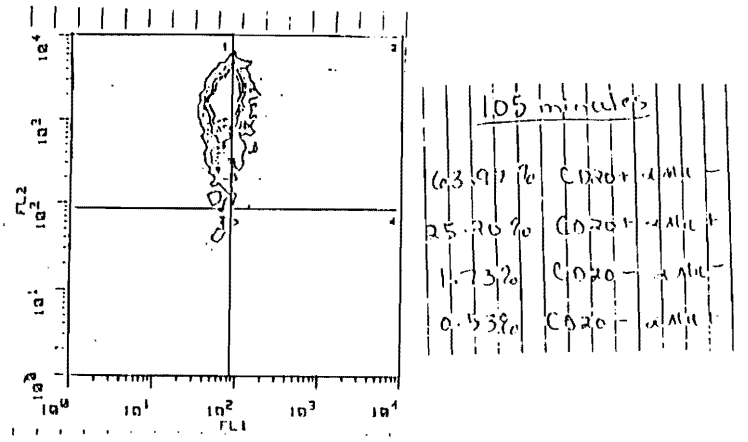
Patching and Capping

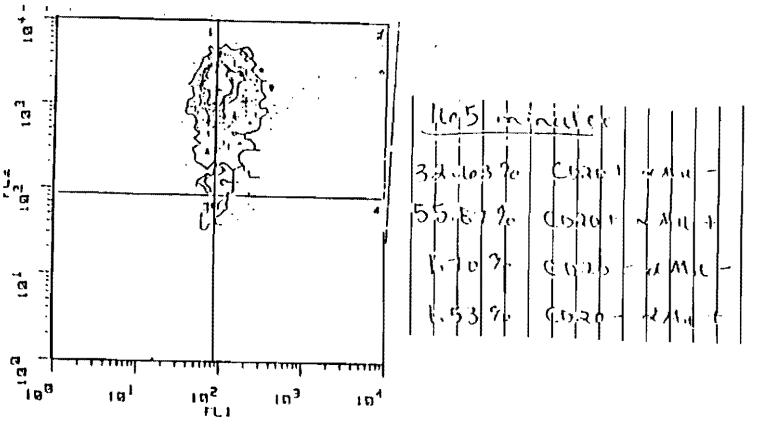
In the anti-hIgM binding experiment, it was shown that around 50% of the cells did bind. In this experiment, binding was only tested at fifteen minutes. Since 100% of the

cells are killed by anti-hIgM, it is of some interest to see if 100% of the cells are ever bound to the anti-hIgM. It was found in an experiment done using four different time points that the percentage of the bound cells shifts in a way that suggests patching and capping is most likely occurring. In the patching and capping process, the anti-hIgM would bind to the cell and then later would be taken inside the cell, while the receptors returned to the surface. This would explain the varying percentage of cells bound to anti-IgM at different time points. This is one possibility that may explain the results. However, this explanation has not been confirmed. There is Sodium Azide present in the monoclonals. Sodium Azide is an inhibitor of the patching and capping procedure since it is an ATP dependent process. The concentration of Sodium Azide present might not have been high enough to prevent this process from occurring. Further analysis will have to be done in order to determine the effect that the Sodium Azide may have had on the process. The following figures show the binding at fifteen, sixty, one-hundred five, and one-hundred sixty-five minutes.



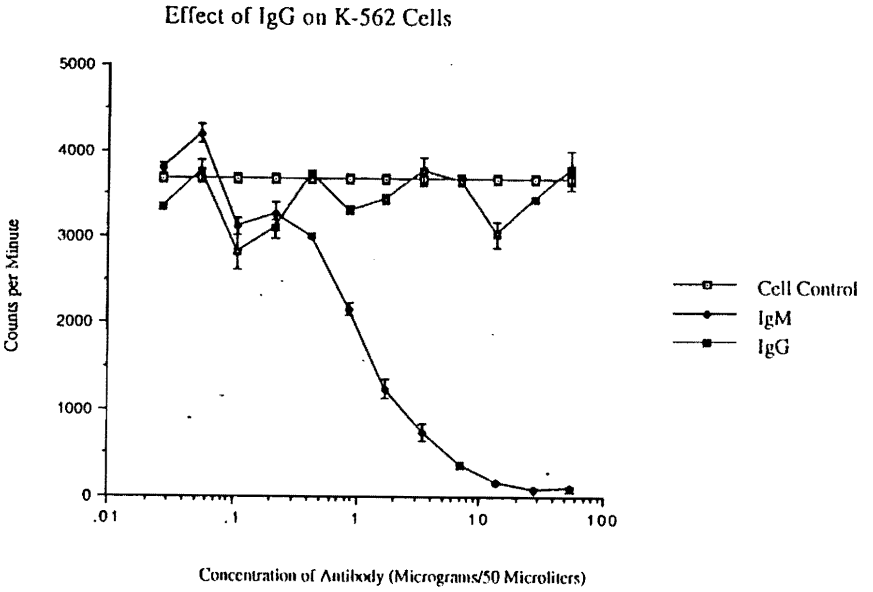






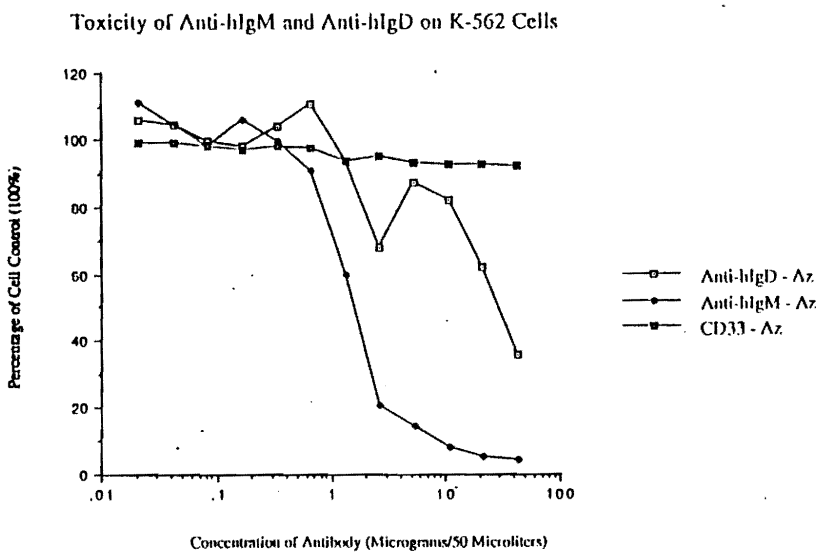
Effect of IgG on K-562 Cells

Since both anti-hIgM and anti-hIgD are toxic to the K-562 cell, another of the Ig molecules was tested to see the effect. IgG did not have an effect on the cell. The following graph shows the results of the IgG experiment.



Effect of Anti-hIgD on K-562 Cells

Anti-hIgD was found to be toxic to the cells. Since both anti-hIgD and anti-hIgM are toxic to the cell, there is a possibility that these cells are immature B cells. Future experiments will determine if the anti-hIgD binds to the cell. The following graph shows the toxicity of anti-hIgD on K-562 cells .



Below is a graph showing the toxicity of both anti-hIgM and anti-hIgD. The anti-hIgM is obviously more toxic to the K-562 cell.