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Effects of the Removal of Adherent and Phagocytic Cells on the Spleen Cell Lymphoproliferative Response of Tumor Bearing Mice

Aileen M. Hyde

Abstract

EFFECTS OF THE REMOVAL OF ADHERENT AND PHAGOCYTIC CELLS ON THE SPLEEN CELL LYMPHOPROLIFERATIVE RESPONSE OF TUMOR-BEARING MICE

by

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Cell-mediated immunity was investigated in two BALB/c mouse tumor systems using the lymphocyte transformation test with phytohemagglutinin as the mitogen. This lymphoproliferative response was quantitated using the Stimulation Index (SI). Spleen cells from mice injected with #51 cells, a chemically-transformed colon cell line, produced a SI value equal to that of normal mice. There was no evidence for suppressor cell activity in cell mixing experiments in which spleen cells from #51 cell-injected mice were mixed with spleen cells from normal mice. Following macrophage removal by Sephadex G-10 columns and carbonyl iron ingestion, there were no significant changes in the SI values for spleen cells from the #51 cell-injected mice. In contrast, spleen cells from mice injected with H238 cells, a herpes virus-transformed cell line, had a significantly lower SI value than that of normal mice. Suppressor cell activity was demonstrated in cell mixing experiments in which spleen cells from H238 cell-injected

mice were mixed with normal spleen cells. Removal of adherent cells from spleen cells from H238 cell-injected mice by Sephadex G-10 columns restored the SI value to that of normal mice. An increased SI value was also seen after removal of phagocytic cells by carbonyl iron. These results suggested that cells with the functional properties of macrophages played an important part in the immuno-suppression observed in the H238 tumor system. Comparison of the two macrophage depletion methods suggested that another cell population was also involved in the suppressive effect.

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Each person whose signature appears below certifies that this manuscript in his opinion is adequate, in scope and quality, in lieu of a thesis for the degree Master of Science.

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INTRODUCTION

Macrophages have been shown to serve at least three functions in tumor immunology. Their selective cytotoxicity to neoplastic cells has been demonstrated (6,20). They also have functioned as accessory cells in other host defense mechanisms (21,60). However, in many tumor systems, macrophages actually suppressed the immune response (1,7,8,10-13,15,17,19,22,23,25-27,29,30,34-36,44-47,50,51,54-57,59,63).

The lymphocyte transformation test (LTT) using the mitogen phytohemagglutinin (PHA) (37) has been used to decipher the role of macrophages. It has been demonstrated that PHA is specifically a T-lymphocyte mitogen and that macrophages are essential for this reaction to occur (14,42,48,49). The LTT has been a useful tool for tumor immunologists as many tumor systems have shown a depressed response that correlated with the immune status of the host and host tumor size (18,22,25,28,41,46,50,52,56,57,59,). Using this test, suppressor macrophages have been indirectly demonstrated in many tumor systems. This was usually accomplished by selectively removing these cells which thereby restored lymphoproliferation to normal levels.

Using the LTT as an assay, suppressor macrophages have been associated with tumors transformed chemically (24,44,46,50,55,59), virally (15,26,27,30,41,57), and

spontaneously (25,56,59). Suppressor macrophages have been demonstrated in spleen cells from tumor-bearing mice (25-27,41,44,46,50,56), rats (22,52,55,57-59) and chickens (53). Using similar protocols suppressor macrophages have been identified in blood from cancer patients (9,29,34,54,63) suggesting potential clinical relevance of these animal studies.

The non-specific action of suppressor macrophages has been indicated by similar results obtained in animal and clinical studies when using in vivo tests (8,11,23,35,36,61) and other in vitro immunological tests that measure the function of T-lymphocytes (1,2,7,10,12,16,17,19,30,33,47,51), B-lymphocytes (1,41,44,45,46,56) and natural killer cells (13).

Splenomegaly has been shown to be a characteristic of many tumor-bearing animals (7,17,24,27,38,41,46,50,62). Of the tumor systems in which suppressor macrophages have been demonstrated, many also have shown splenomegaly (7,17,41,46,50,62). These include cell lines transformed by viruses (17,41) and chemicals (7,46,50,62).

Our laboratory has been studying two BALB/c mouse tumor systems; one in which tumors are produced by injection of chemically-transformed cells (#51), and the other in which the tumors are produced as a result of injection of virally-transformed cells (H238) (18,38-40). With both

systems, tumor-bearing mice showed splenomegaly. The splenomegaly was more pronounced in the H238 system, with the relative spleen weight six times greater than normal (18); whereas in the #51 system, the spleens were twice the size of normal spleens (40). Furthermore, in the H238 system, there was a depressed lymphoproliferative response to PHA correlating with tumor progression. Spleen stereology studies with H238 system showed an increase in the compact myeloid tissue, lymphoid nodule reaction centers, and red pulp volume (38). Suppressor macrophages have been demonstrated in another tumor system in which there was also in increased red pulp volume (24). In order to determine if suppressor macrophages were acting in the BALB/c mouse: H238 tumor cell system and in the BALB/c mouse: #51 tumor cell system, the effects of macrophage removal from spleen cells of tumor-bearing mice on the lymphocyte transformation test were monitored.

MATERIALS AND METHODS

Mice

BALB/c mice were bred at our institution from mice originally purchased from The Jackson Laboratory (Bar Harbor, ME). The offspring were housed in groups of 6-8 in shoebox-type cages and fed Purina rodent laboratory chow and tap water *ad libitum*. At the time of injection with tumor cells, the mice were 18-20 weeks of age.

Tumor Systems

The H238 tumor was derived from a BALB/c mouse embryo skin cell line (238) transformed by ultraviolet light-irradiated herpes simplex virus type 2 (Savage strain) (3). The #51 tumor was derived from BALB/c mouse colon cells transformed *in vivo* by 1,2-dimethylhydrazine (4) and adapted for cell culture in our laboratory. The cells were stored in liquid nitrogen and grown in Dulbecco's medium supplemented with 10% fetal bovine serum before injection. The mice in each cage were divided into three groups and identified by marking with picric acid. By subcutaneous injection in the right thigh, one group received 1×10^6 H238 cells in 0.3 ml tris-buffered saline (TBS), another group 1.6×10^5 #51 cells in 0.3 ml TBS, and the control group was injected with 0.3 ml TBS only. At the time of sacrifice seven weeks later, all but two tumor-cell injected

mice had tumors. These two were excluded from the study. There was no evidence of tumors in the control mice.

Lymphocyte Transformation Test

For each experimental group, 2×10^5 viable spleen cells were cultured in eight replicate flat-bottom wells of a microtiter plate (Costar, Cambridge, MA). The culture medium consisted of RPMI-1640 supplemented with 20 mM HEPES buffer, 5μM 2-Mercaptoethanol, 10% heat-inactivated fetal bovine serum (56°C for 30 minutes), 100 U/m penicillin, 4 mg/ml Streptomycin, 5 μg/ml Fungizone and 1 U/ml Mycostatin. The four test wells received 0.28 μg of PHA (Burroughs Wellcome Co., Research Triangle Park, NC) in 0.1 ml RPMI-1640, whereas the four control wells received 0.1 ml of RPMI-1640. Five hours prior to culture termination, 1.0 microcurie of ^{3}H-thymidine (Research Products International Group, Mt. Prospect, IL, Spec. Act. 49.5 Ci/mM) in 0.05 ml RPMI-1640 was added to each well. After 48 hours of culture, the cells were harvested with a multiple sample harvester (Brandel, Gaithersburg, MD) and counted for 2 minutes in a liquid scintillation counter (Beckman, Los Angeles, CA). The Stimulation Index (SI) was calculated from the counts per minute (CPM) according to the following formula:

$$\text{Stimulation Index} = \frac{\text{Test CPM} - \text{Control CPM}}{\text{Control CPM}}$$

in which Test CPM represents average values for those wells which received PHA and Control CPM represents average values for those wells which did not receive PHA.

Removal of Adherent Cells by Sephadex G-10 Columns

The standard method (32) was adapted to use 12 ml disposable syringes as columns. A Sephadex G-10 slurry in 0.85% saline was added to each syringe to give a 10 ml bed volume. The column was then rinsed with 25 ml of warm RPMI -1640 medium. A 2 ml suspension containing 1 x 10^8 viable spleen cells was added to the column and rinsed through with 10.0 ml RPMI-1640.

Removal of Phagocytic Cells by Carbonyl Iron Ingestion

Viable spleen cells (1 x 10^8) from tumor-injected mice were suspended in 10 ml RPMI-1640 containing 100 mg carbonyl iron (Sigma, St. Louis, MO). After one hour of incubation at 37°C in a 5% CO_2 incubator, cells containing carbonyl iron and excess carbonyl iron were retained with a 6 lb. magnet and the remaining cells pipetted off. This process was repeated 2-3 times.

Enrichment of Adherent Cells by Petri Dish Adherence

Viable spleen cells (5 x 10^6) were suspended in 2 ml RPMI-1640 and plated into a 60 x 15 mm tissue culture dish (Corning Glass Works, Corning, NY). Following a one

hour incubation at 37°C in a 5% CO_2 atmosphere, the nonadherent cells were pipetted off and the adherent cells were removed by scraping with a rubber policeman.

Spleen Cell Differential Counts

After straining with Wright's stain, a 100-cell differential count was done and the cells were classified as lymphocytes, granulocytes, or macrophages.

Statistical Analysis

The CPM values obtained from the lymphocyte transformation tests were subjected to the r method (5) of excluding outliers prior to calculation of the means for the stimulation index.

The two-sided Student's t-test was used to compare groups. The $p=0.05$ level was chosen as the level of significance.

RESULTS

Spleen Cell SI Values

The spleen cells from the H238 cell-injected mice produced lower SI values than their normal counterparts in LTT assays; however, the spleen cell response of the #51 cell-injected mice, though lower, was not significantly different from the normal response (Table 1). Comparing the spleen cell differential counts of normal mice with H238 cell-injected mice showed an increase in granulocytes and macrophages ($p=0.05$) whereas the #51 cell-injected mice showed an increase only in the granulocyte population ($p=0.05$) (Table 2).

Cell Mixing Experiments

The ability of spleen cells to suppress the lymphoproliferative response of normal cells was examined in cell-mixing experiments. Spleen cells from #51 cell-injected mice were not suppressive as there were no significant differences in the SI values of various combinations of spleen cells from normal and #51 cell-injected mice (Figure 1). In contrast, spleen cells from H238 cell-injected mice suppressed the SI values of normal spleen cells (Figure 2). The only exception was the mixture containing 90% cells from normal mice and 10% cells from H238 cell-injected mice in which the SI value was not significantly different than that of 100% spleen cells from normal mice.

Effects of Macrophage Removal and Reconstitution in the H238 System

Spleen cells from H238 cell-injected mice, after passage through the Sephadex G-10 column, produced a SI value equal to that of normal spleen cells (Figure 3). Spleen cell differential counts showed that passage through Sephadex G-10 columns resulted in depletion of macrophages and granulocytes (Table 2). The reconstitution part of the experiment was designed to test for the presence of suppressor macrophages. The hypothesis was that the addition of an enriched macrophage population to macrophage-depleted cells would return the SI value to that of untreated spleen cells from H238 cell-injected mice. Adding back adherent spleen cells from both normal and H238 cell-injected mice to the Sephadex G-10 nonadherent cells did not drop the SI value to that equal to untreated cells (Figure 3). Spleen cell differential counts showed that there were no significant changes between the untreated population of spleen cells and the adherent spleen cells from both normal and H238 cell-injected mice (Table 2). As shown in Figure 4, carbonyl iron nonphagocytic spleen cells from H238 cell-injected mice had an elevated SI value not statistically different than that of normal cells but not significantly greater than that of the untreated population. Reconstituting the carbonyl iron nonphagocytic cells with normal

adherent spleen cells did not change the SI value from that of carbonyl iron nonphagocytic cells, whereas adding adherent spleen cells from H238 cell-injected mice lowered the SI value to that of the untreated population (Figure 4). Spleen cell differential counts showed that carbonyl iron treatment resulted in depletion of granulocyte and macrophage populations (Table 2).

Effects of Macrophage Removal and Reconstitution in the #51 System

There were no significant changes in the response of spleen cells from #51 cell-injected mice following Sephadex G-10 treatment and reconstitution with adherent cells (Figure 5) or following carbonyl iron treatment with subsequent reconstitution with adherent cells (Figure 6).

DISCUSSION

Spleen cell differential counts from both groups of tumor-bearing mice showed a decreased percentage of lymphocytes in reference to normal mice. Both groups of tumor-bearing mice demonstrated splenomegaly. This splenomegaly was quantitated by determining the number of cells recovered per spleen. Compared to the number of spleen cells from normal mice, #51 cell-injected mice showed a three-fold increase and H238 cell-injected mice showed a seven-fold increase in number of cells. Furthermore, changes in spleen morphology in H238 cell-injected mice have been previously characterized by stereology studies in which it was shown that the splenomegaly was not due to increases in splenic connective tissue (38). Thus the decreased percentage of lymphocytes was due to a relative change in percentage of lymphocytes rather than an absolute decrease in total lymphocyte numbers.

The SI values of spleen cells from mice with the chemically-induced tumor, #51, were not statistically different from those of normal mice. It was not surprising then that there were no differences between the mixtures and starting populations in the cell mixing experiments. Techniques used to remove and add back macrophages had no effect on the SI values produced by spleen cells from #51

cell-injected mice. With this assay, there was no evidence for suppressor macrophages in the #51 system. One cannot be certain, however, that suppression by macrophages was not involved in the #51 cell-injected tumor system. Since suppressor macrophages have been demonstrated in other chemically-induced tumor systems (7,46,50,62), it might be that the LTT assay system used was not sensitive enough to detect differences in the response of spleen cells from these mice compared with those from normal mice.

In contrast, the SI values produced by spleen cells from mice bearing the virally-induced tumor, H238, was less than that of normal mice. To rule out that this suboptimal response of spleen cells from H238 cell-injected mice was an artifact resulting from a tumor-induced shift in the dose-response curve, PHA titrations with spleen cells from both normal and H238 cell-injected mice were done. Figure 7 shows that the SI values obtained depicted a bell-shaped curve as established in earlier work (31) and that the optimal concentration of PHA was the same for both sets of spleen cells.

Cell mixing experiments have been used to detect active suppression from cells of tumor-bearing animals (41,44,46,56). Spleen cells from H238 cell-injected mice suppressed the lymphoproliferative response of normal cells (Figure 2). The data suggests that a critical number of

spleen cells from tumor-bearing animals were necessary for the suppression to occur. Suppression of the SI value of normal cells occurred only when spleen cells from H238 cell-injected mice constituted 25% and 50% of the culture; whereas there was no suppression when the culture contained only 10% spleen cells from H238 cell-injected mice.

When the spleen cell differential counts of H238 cell-injected mice were compared with those of normal mice, the greatest change was found to be in the increase in percentage of granulocytes in the tumor-bearing mice. A likely candidate for a suppressor cell is a cell type increased in an immunosuppressed tumor-bearing animal. An unexpected finding in this series of experiments was that the techniques chosen for macrophage removal were also effective for granulocyte depletion (Table 2). One can argue against granulocytes acting as suppressor cells in the H238 system by comparing our two tumor systems in which both showed granulocyte infiltration associated with tumor-bearing but immunosuppression only in the H238 system. There have been no reports in the literature of granulocyte-mediated suppression.

Macrophage removal techniques increased the SI values produced by spleen cells from H238 cell-injected mice. Cells that did not adhere to Sephadex G-10 columns produced a higher SI value than cells that did not

phagocytize carbonyl iron (Table 1). The only significant difference between these groups in the spleen cell differential counts was the percentage of macrophages (Table 2). One interpretation of this data would be that all the macrophages functioning as suppressor cells have been removed in both methods. Sephadex G-10 columns have been shown to remove suppressor T lymphocytes (43). The augmented response of the Sephadex G-10 non-adherent cells might be due to the removal of another type of suppressor cell.

In our reconstitution experiments, adding adherent cells back to cells that have been passed through Sephadex G-10 columns did not reduce the SI value. This protocol has been used by others to confirm that macrophages are functioning as suppressor cells (56). However, these investigators reported an adherent spleen cell population consisting of 95-99% macrophages. In contrast, we did not achieve an enriched macrophage population from our starting population in any of the three groups of mice (Table 2). With improved techniques yielding a higher percentage of macrophages, we would expect to see the SI values of both spleen cells of normal mice and Sephadex G-10 nonadherent spleen cells from H238 cell-injected mice reduced to that of spleen cells from H238 mice. Spleen cells from H238 cell-injected mice that did not ingest carbonyl iron

responded quite differently to reconstitution with adherent cells (Figure 4). When normal adherent cells were added, there was no change in the SI value compared to that of the carbonyl iron treated cells; whereas when adherent spleen cells from H238 cell-injected mice were added the SI value was reduced to that equal to the starting population.

Comparison of the SI values of the reconstituted cultures from macrophage-depleted spleen cells of H238 cell-injected mice suggests a specific cellular interaction was needed for immunosuppression to occur. Figure 8 shows that of the four groups only the carbonyl iron treated cells reconstituted with adherent cells from spleens of H238 cell-injected mice produced SI values significantly lower than that of 100% normal spleen cells. There was no suppression of the carbonyl iron nonphagocytic cells when reconstituted with adherent spleen cells from normal mice; but a significant depression when the same carbonyl iron treated cells were reconstituted with adherent spleen cells from H238 cell-injected mice. This suggests that a tumor-induced adherent suppressor cell played a role in the immunosuppression. Comparing the response of cells not adhering to Sephadex G-10 columns, there was no difference between the SI values produced by cultures reconstituted with adherent cells from normal and H238 cell-injected mice. A possible explanation for the difference between the

response of reconstituted cultures from the two macrophage depletion methods is that the cell mediating the suppression was removed by the Sephadex G-10 columns. The carbonyl iron method removes phagocytic cells, i.e. granulocytes and macrophages, whereas the Sephadex G-10 columns remove adherent cells, i.e. macrophages, and nonspecifically T suppressor cells (43). Since macrophage removal was common to both methods, T suppressor cells seem to be a likely candidate for the cell interacting with the adherent cells from H238 cell-injected mice to produce the immunosuppression in the H238 system.

The cellular mechanism for the suppression of the lymphoproliferative response of H238 cell-injected mice is consistent with tumor-induced adherent cell, presumably a macrophage, interacting with T suppressor cells. Investigations to test this model are now underway in our laboratory.

TABLE 1. STIMULATION INDEX OF EXPERIMENTAL GROUPS

Results of Five Replicate Experiments

	Experimental Group	Mean	Standard Error
	Baseline:		
1.	Spleen Cells from normal Mice (N)	9.7	2.3
2.	Spleen Cells from H238 cell-injected Mice (238)	0.8	0.1
3.	Spleen Cells from #51 cell-injected Mice (#51)	3.9	1.1
	Cell Mixing Studies:		
4.	50% N & 50% H238	3.3	0.7
5.	75% N & 25% H238	3.3	1.0
6.	90% N & 10% H238	5.4	1.3
7.	50% N & 50% #51	5.4	1.3
8.	75% N & 25% #51	3.6	0.4
9.	90% N & 10% #51	5.8	2.0
	Macrophage Studies: All wells contain 50% N plus:		
10.	50% Carbonyl Fe Nonphagocytic H238 (CF-H238)	6.3	1.7
11.	40% CF-H238 & 10% Normal Adherent	7.3	2.6
12.	40% CF-H238 & 10% H238 Adherent	3.5	1.0
13.	50% Sephadex G-10 Nonadherent H238 (G-H238)	8.9	1.8
14.	40% G-H238 & Normal Adherent	6.9	1.9
15.	40% G-H238 & 10% H238 Adherent	8.9	2.4
16.	50% Carbonyl Fe Nonphagocytic #51 (CF-#51)	6.3	1.8
17.	40% CF-#51 & 10% Normal Adherent	6.6	2.0
18.	40% CF-#51 & 10% #51 Adherent	6.1	1.5
19.	50% Sephadex G-10 Nonadherent #51 (G-#51)	4.2	1.3
20.	40% G-#51 & 10% Normal Adherent	5.4	2.1
21.	40% G-#51 & 10% #51 Adherent	6.6	1.7

TABLE 2: Spleen Cell Differential Counts (mean ± standard error)

Group	% Lymphocytes	%Granulocytes	% Macrophages
H238 Cell-injected Mice: (H238)			
Untreated	45.2±5.5	46.0±8.3	8.8±3.1
Sephadex G-10 Nonadherent (G-H238)	84.6±3.9	13.6±3.5	1.8±0.8
Carbonyl Fe Nonphagocytic (CF-H238)	79.2±7.8	20.5±8.0	0.3±0.3
Petri Dish Adherent (H238 Adherent)	55.7±3.5	35.7±3.5	8.6±0.5
#51 Cell-injected Mice: (#51)			
Untreated	82.3±4.1	16.7±3.9	1.0±0.5
Sephadex G-10 Nonadherent (G-#51)	99.8±0.6	0.2±0.2	0± 0
Carbonyl Fe Nonphagocytic (CF-#51)	98.2±0.5	1.8±0.5	0± 0
Petri Dish Adherent (#51 Adherent)	93.7±3.4	3.0±1.0	3.3±2.4
Normal:			
Untreated	93.4±0.9	5.3±1.3	1.3±0.5
Petri Dish Adherent (Normal Adherent)	95.3±3.7	3.7±3.2	1.0±0.6

FIGURE 1: Cell Mixing Experiment Using Spleen Cells from #51 Cell-injected and Normal Mice

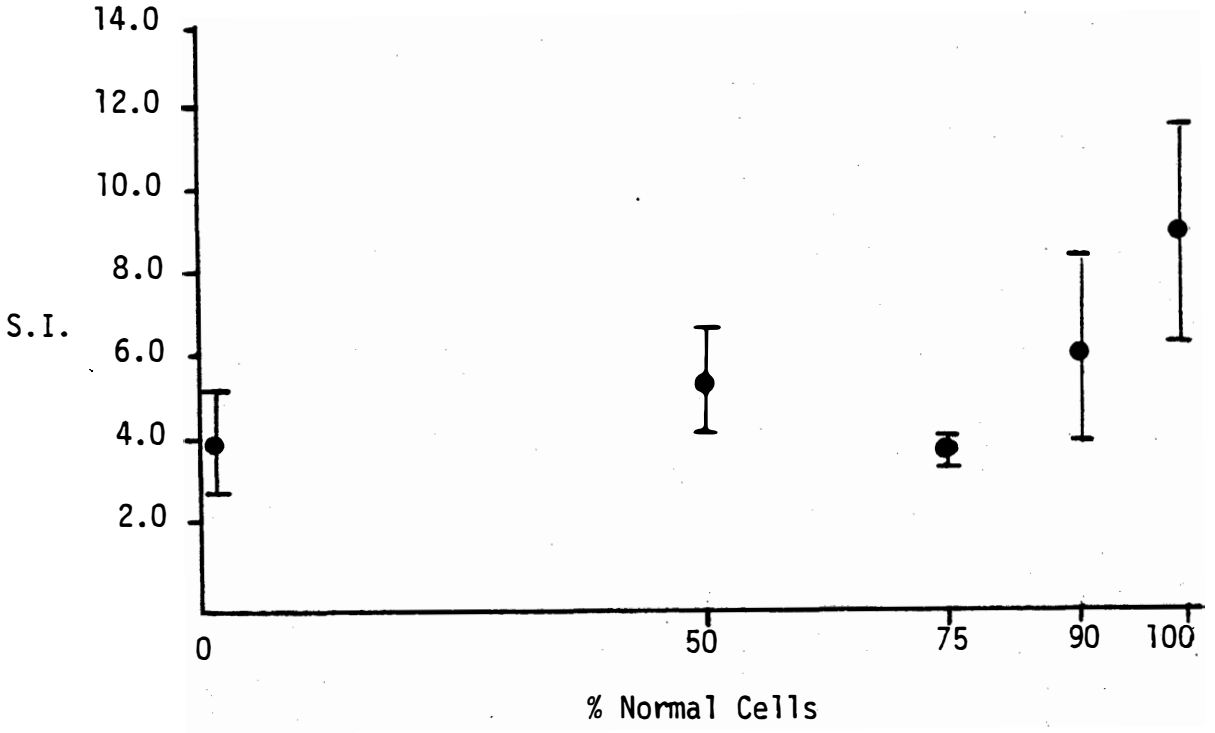


FIGURE 2: Mixing Experiment Using Spleen Cells from H238 Cell-injected and Normal Mice

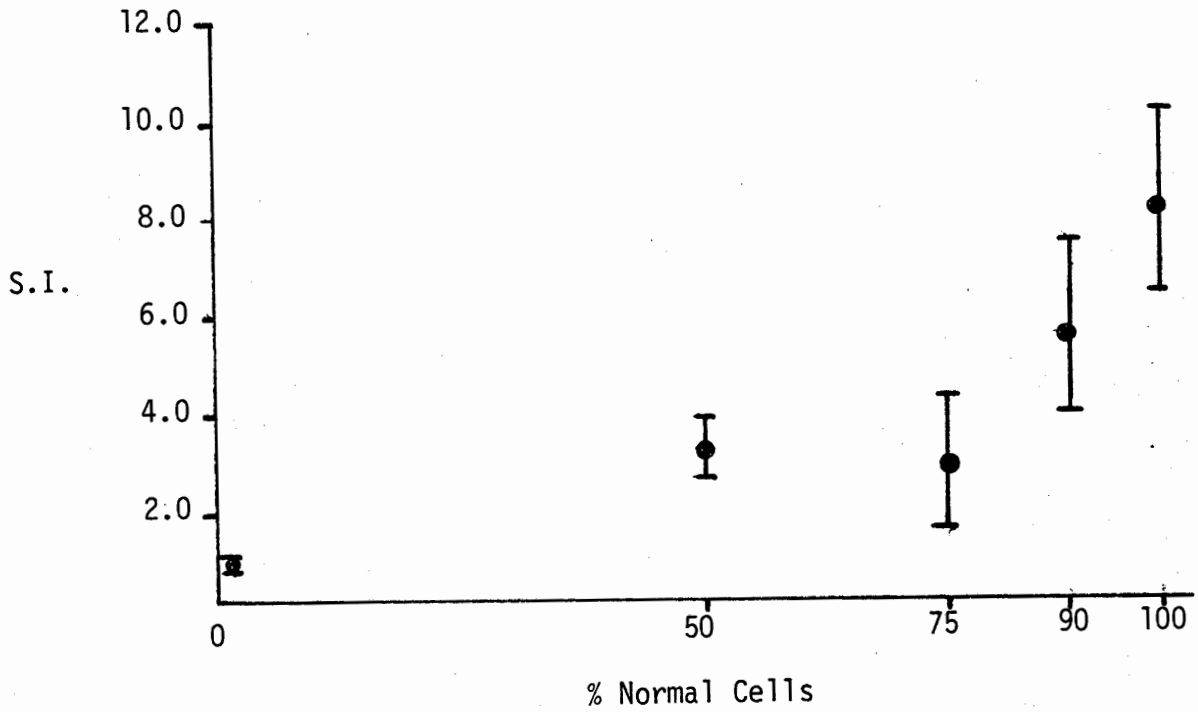


FIGURE 3: Effects of Sephadex G-10 Treatment and Reconstitution with Adherent Cells on Spleen Cell Response to PHA Stimulation in the H238 System

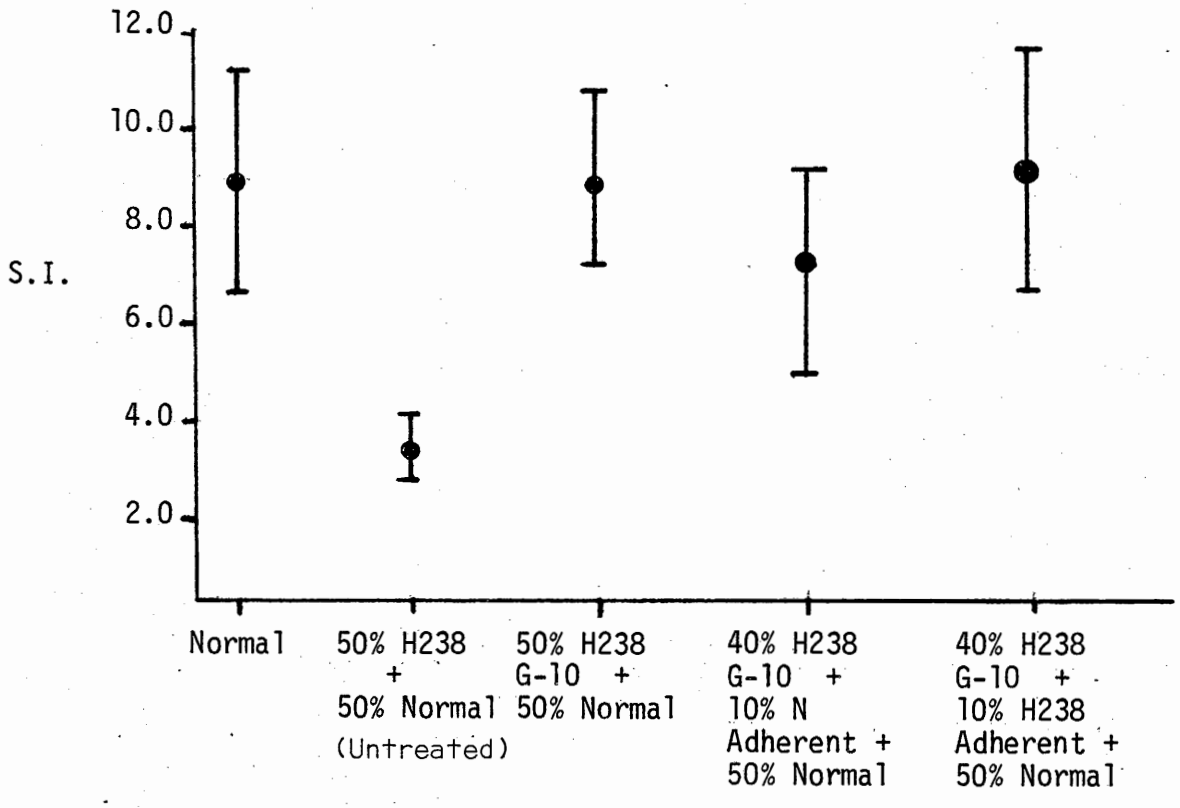


FIGURE 4: Effect of Carbonyl Iron Treatment and Reconstitution with Adherent Cells on Spleen Cell Response to PHA Stimulation in the H238 System

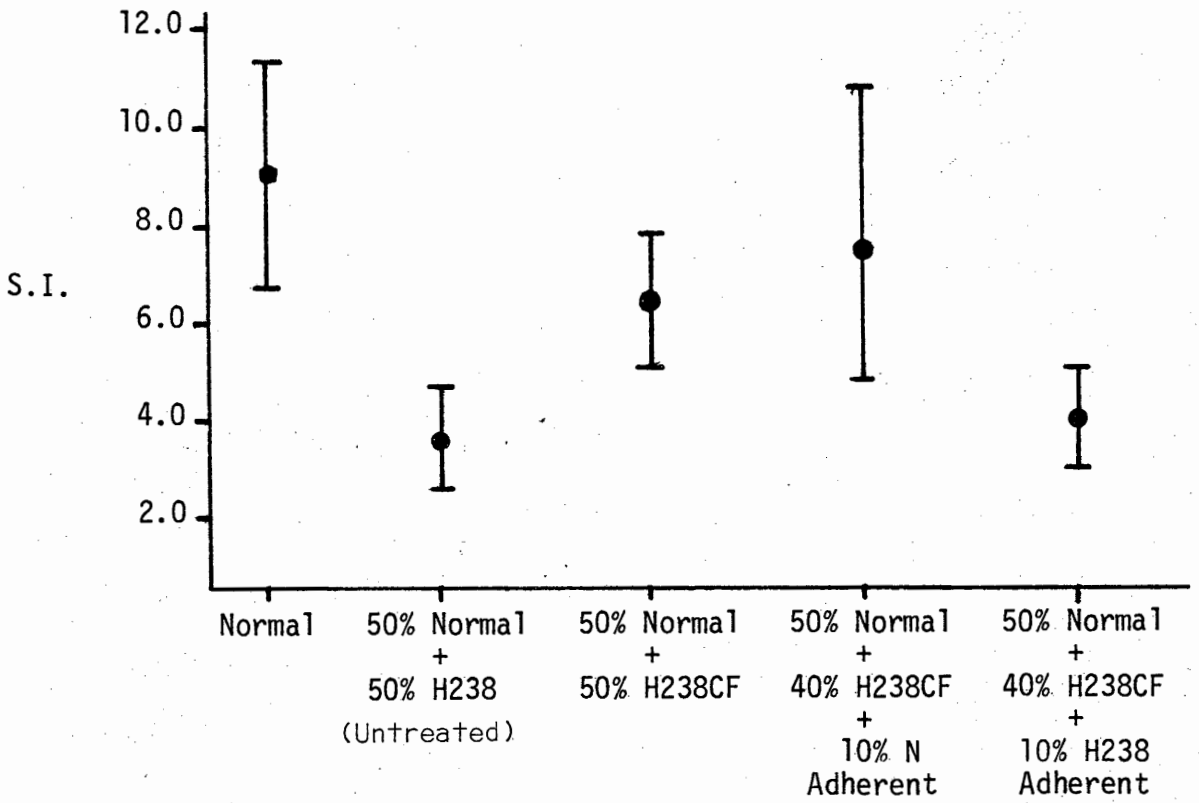


FIGURE 5: Effects of Sephadex G-10 Treatment and Reconstitution with Adherent Cells in Spleen Cell Response to PHA Stimulation in the #51 System

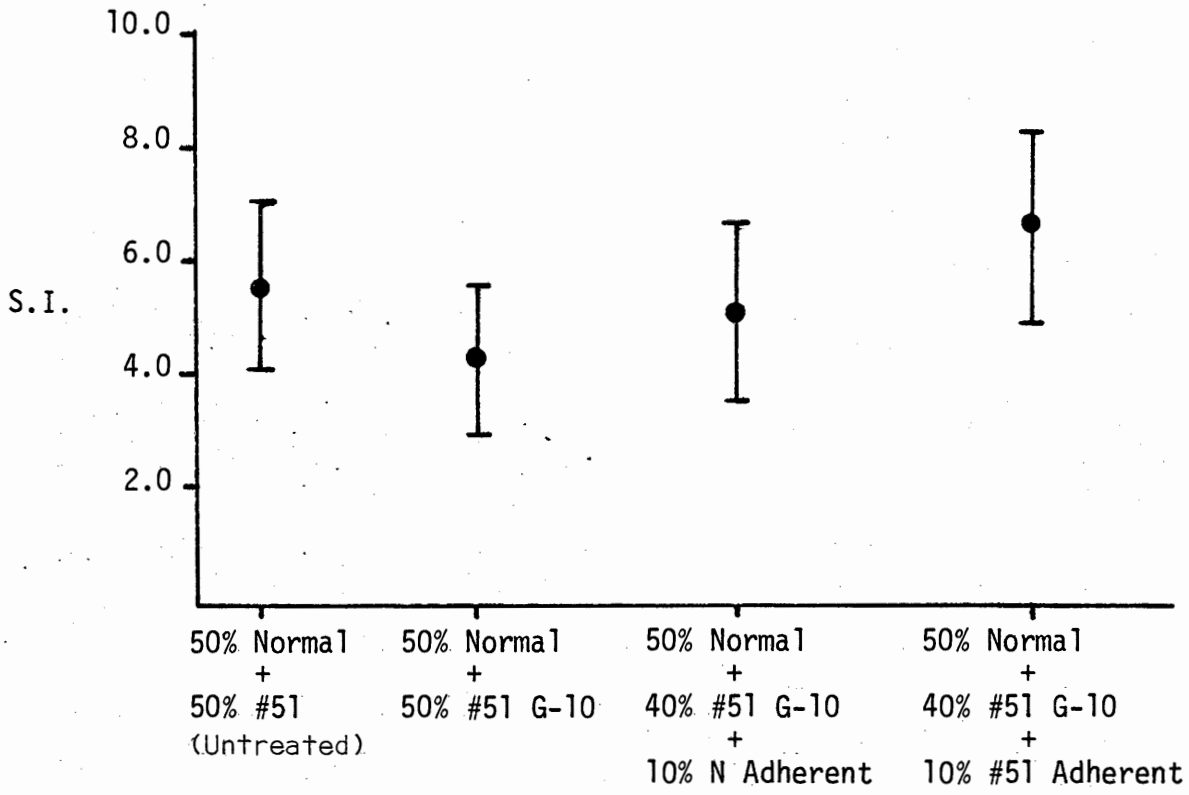


FIGURE 6: Effects of Carbonyl Iron Treatment and Reconstitution with Adherent Cells on Spleen Cell Response to PHA Stimulation in the #51 System

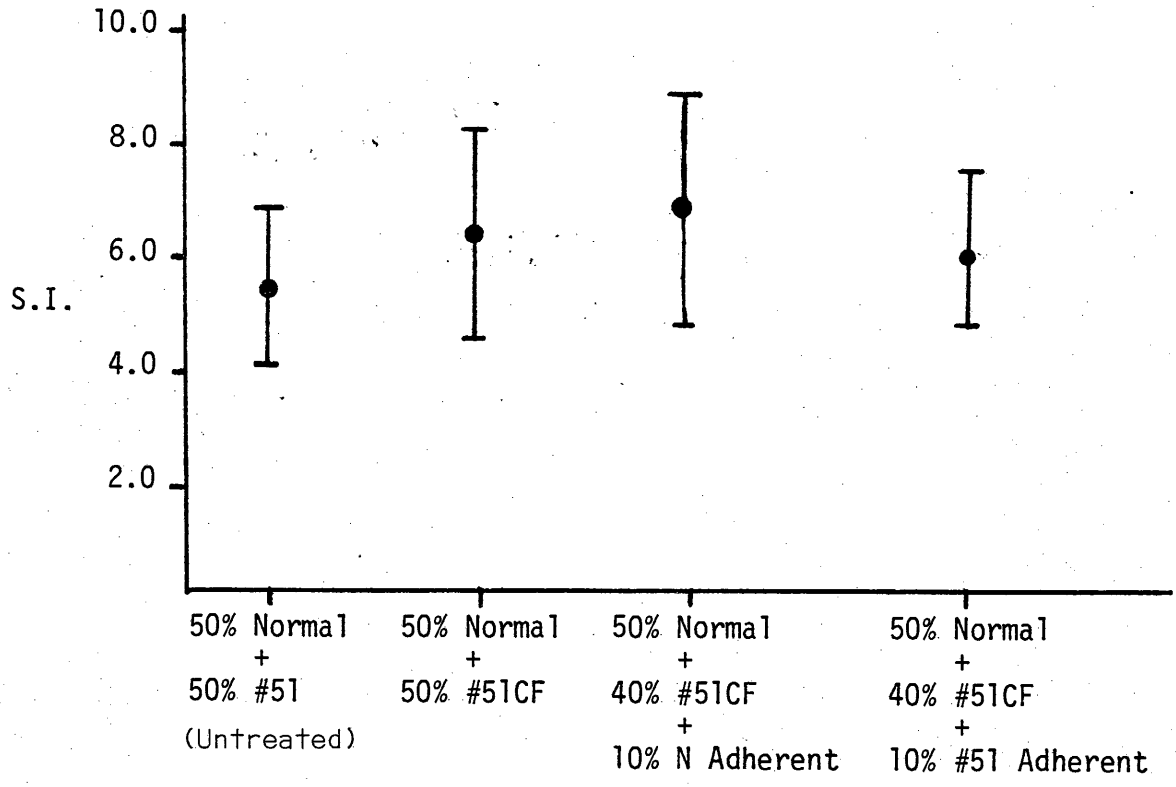


FIGURE 7: PHA Dose Response Curves
Spleen Cells from Normal and
H238 Cell-Injected Mice

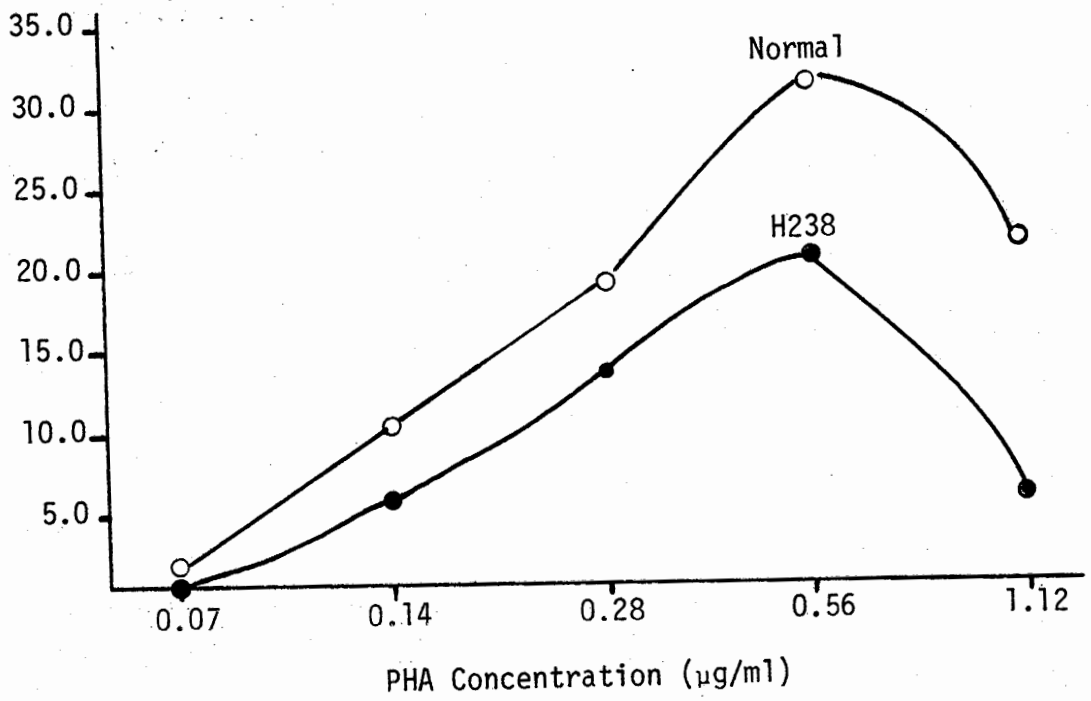
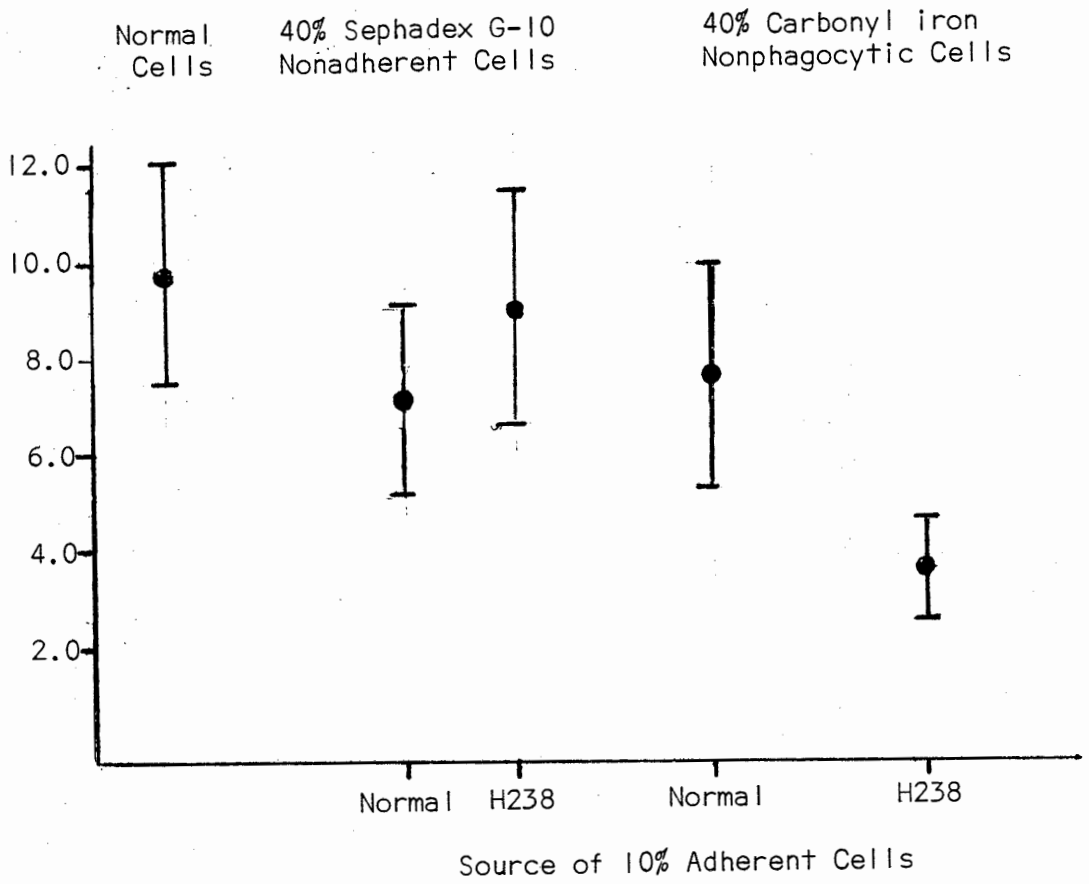


FIGURE 8: Effect of Reconstitution with Adherent Cells on Spleen Cell Response to PHA Stimulation in the H238 System



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