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Title:

Separation of Lymphocytes by Electrophoresis
Under Terrestrial Conditions and at
Zero Gravity

Phase III:

Annual Report
1/1/77 - 12/31/77

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PHASE III

Contract #NAS 9 - 15378

TWELVE MONTH SUMMARY REPORT

1/1/77-12/31/77

SCIENTIFIC PART

PART I. STUDIES ON LYMPHOCYTE ELECTROPHORESIS

A. Electrophoretic Mobility of Fractionated Peripheral Lymphocytes from Normal Adults, Hemodialysis Patients and Kidney Transplant Recipients.

Introduction

B lymphocyte is one of the major subpopulations of lymphocytes, and this cell, as a precursor of plasma cell, does play a pivotal role in an organ transplantation either to reject the graft by production of cytotoxic antibodies or adapt the graft by production of enhancing antibodies. Despite this, B lymphocyte has received less attention than other lymphocyte subpopulations from the transplantation immunologists, probably because of limited biological means to study immunobiological function of B lymphocytes.

To evaluate the potential of lymphocyte electrophoresis in predicting the outcome of kidney transplants and as a method for separation of lymphocyte subpopulations, we studied electrophoretic mobilities (EPM) of peripheral lymphocytes, fractionated (T and non-T lymphocytes) and nonfractionated, from normal subjects, chronic hemodialysis patients and kidney transplant recipients previously. The conclusions resulting from the previous study were: 1) EPM of non-T lymphocytes from chronic maintenance hemodialysis patients and kidney transplant recipients is significantly decreased from normal, and transplant recipients exhibit the greatest change, and 2) a series of experiments suggest that decreased EPM of non-T lymphocytes in kidney transplant recipients is due to actual alterations in proportions of subsets of non-T lymphocytes and/or alterations in functional status of existing non-T lymphocyte subsets as a response to either kidney transplantation per se or immunosuppressive therapy. Since the lymphocytes which do not form spontaneous rosette formation with sheep erythrocytes, referred to as "non-T lymphocytes" in that study are heterogenous with regard to cell type (B lymphocytes, null

cells and possibly other cell types), the observation of decreased EPM of non-T lymphocytes from hemodialysis patients and kidney transplant recipients is actually a net result of the combined EPM of B lymphocytes and null cells. Thus, the results raised new questions: which subpopulation of the non-T lymphocytes is primarily affected and is shown a decreased EPM in chronic hemodialysis patients and kidney transplant recipients? Are they B lymphocytes or null cells, or both?

We developed a technique to separate B lymphocytes and null cells from non-T lymphocyte preparation to answer these questions and conducted a series of new experiments studying EPM of T, B and null cells from normal subjects (N = 5), chronic hemodialysis patients (N = 5) and kidney transplant recipients (N = 11).

Materials and Methods

Materials

EPM of T, B lymphocytes and null cells were studied from 5 normal adult subjects, 5 patients receiving chronic maintenance hemodialysis and 11 kidney transplant recipients. Criteria for selection of study subjects are identical with those which were used for the previous study (Phase I and Phase II). All subjects were fully informed of the study and the patients who participated in this study were treated exactly the same as the patients who did not participate in the study.

Normal subjects' ages ranged from 24 to 40 years, and all were females except one. The uremic subjects' ages ranged from 40 to 64 years; 3 were males and all had been on hemodialysis treatment for at least one year prior to the study. Ages of the kidney transplant recipients ranged from 20 to 57 years and 9 were males. Seven of these patients received renal allografts 3 to 26 days prior to this experiment (Short-term Study), and were on immunosuppressive therapy consisting of 50 to 200 mg of prednisone and 100 to 200 mg of azathioprine per day. The remaining 4 kidney transplant recipients received renal allografts 6 months to 5 years prior to this experiment (Long-term Study), and were also on immunosuppressive drugs, but were receiving lower doses (prednisone 10 to 20 mg and azathioprine 100 mg per day). Although

all of the patients in the Long-term Study group had good and stable graft function, 2 of the 7 patients in the Short-term Study group had no significant graft function at the time of the study and 2 of these patients subsequently lost their grafts due to irreversible graft rejection.

Method

Separation of lymphocytes.

50 to 80 ml of freshly drawn venous blood was mixed with preservative-free heparin, layered on a Ficoll-Hypaque gradient, and lymphocytes were separated after centrifugation. To remove monocytes and neutrophilic leucocytes, the lymphocyte preparation was suspended in Hank's Balanced Salt Solution (HBSS) with 10% heat inactivated (decomplemented) human AB serum and incubated on Falcon plastic petri dishes for 1 hour at 37°C in 5% CO₂ - 95% O₂. This procedure usually provides over 90% pure and over 90% viable lymphocytes. (The details of this procedure were described in the previous report.)

Separation of T lymphocytes (E rosetting).

Sheep erythrocytes (SRBC) were washed twice in HBSS, and three drops of packed SRBC were suspended in 10 ml HBSS. Equal volumes of human lymphocytes (2×10^6 lymphocytes/ml RPMI 1640 + 10% AB serum + 1% glutamine) and SRBC suspension were combined in Falcon plastic tubes, and the tubes were centrifugated at 500 rpm for 5 minutes at 25°C. The cells were incubated overnight (18 hours) at 4°C. The following day, the pellets were gently resuspended and were layered over 12 ml Ficoll-Hypaque and centrifugated at 1,400 rpm for 40 minutes at 4°C. Unrosetted lymphocytes were removed from the interface. Rosetted T lymphocytes in the pellet beneath the Ficoll-Hypaque were washed with ammonium chloride tris buffer to lyse SRBC and washed again with HBSS + 10% AB serum.

Separation of B lymphocytes and null cells (E.A.C. rosetting.)

The lymphocyte preparation which did not form rosettes with SRBC is referred to as "non-T lymphocytes preparation" and contains predominantly B lymphocytes, as well as null cells and a few monocytes. The "non-T lymphocyte preparation" was further processed to separate primarily B lymphocytes from null cells.

SRBC were washed twice in Dulbecco's PBS (Grand Island Biological Co., Grand Island, N.Y.) prior to the incubation with rabbit anti-SRBC antibody (Cappel Laboratories, Inc., Downingtown, Pa.). Equal volumes of a 5% SRBC solution and a 1/1000 dilution of the antibody were combined and incubated for 30 minutes at 37°C. Following the incubation, the SRBC-antibody (E.A.) was washed twice in HBSS. The E.A. complex was resuspended in a 5% solution and combined in equal volumes with a 1/20 dilution of fresh human serum, and the mixture was incubated for 30 minutes at 37°C followed by two washes with HBSS. The SRBC-antibody complement (E.A.C.) was resuspended in 1% solution with HBSS. Equal volumes of the E.A.C. suspension and the "non-T lymphocyte preparation" (4×10^6 cells/ml in RPMI 1640 and 10% FCS) were incubated at 37°C for 30 minutes following a centrifugation at 1000 rpm for 5 minutes.

After incubation, the pellet was resuspended by hard mixing and layered over Ficoll-Hypaque. Prior to layering, ratio of 100 cells with and without rosettes was counted. The layered suspension was centrifugated for 15 minutes at 20°C 1000 rpm. The non-rosetted cells, designated as null cells were removed from the interface and washed twice with HBSS. The rosetted B lymphocytes from a pellet were treated with ammonium chloride Tris buffer to lyse the SRBC and washed twice with Ca and Mg free HBSS and 10% FCS.

Electrophoresis.

Electrophoretic mobility of lymphocytes was determined using Cylindrical Microelectrophoresis Apparatus (Type U2, Rank Brothers, Bottisham, Cambridge, England) with suspension media of 0.03 mol. liter⁻¹ ionic strength, 4.0 gm/dl sorbitol, buffered to pH 7.4 $\pm$ 0.2 with sodium bicarbonate, at 25°C. Fresh human erythrocytes were used as a reference material before and after each measurement of lymphocyte electrophoretic mobility. Each one hundred of T, B and null cells were studied in each subject.

Results

Ranges and means ($\pm$ SEM) of EPM of T, B and null cells from normal subjects, chronic hemodialysis patients and kidney transplant recipients are shown in Table 1. Also, frequency polygons of EPM produced from the results are displayed in Figures 1 to 6 to compare the differences between groups of study subjects in the same lymphocyte subpopulation and between lymphocyte subpopulations in the same group of study subjects.

In normal subjects, T lymphocytes exhibited highest EPM and null cells exhibited lowest RPM, and the difference of EPM between null cells from T ($P < 0.03$) were statistically significant. In chronic hemodialysis patients, EPM of both of the T ($P < 0.03$) and B lymphocytes ($P < 0.01$) were significantly decreased from EPM of respective lymphocytes from normal subjects, and EPM of B lymphocytes was so low that EPM of B and null cells were identical. In kidney transplant recipients, EPM of T ($P < 0.003$) and B lymphocytes ($P < 0.01$) were again significantly lower than those of normals, and B lymphocyte EPM approached to null cell EPM. However, there was no difference in EPM of all subpopulations of lymphocytes between short and long-term post-transplant patients, and between hemodialysis patients and kidney transplant recipients. EPM of null cells was identical among the normal subjects, hemodialysis patients and kidney transplant recipients.

Comments

In the previous report, we had shown that the EPM of non-T lymphocytes in hemodialysis patients and kidney transplant recipients, is significantly decreased than that of normal subjects, and that the transplant recipients exhibited the greatest changes. The low EPM in transplant recipients, however, was poorly correlated with either duration of transplantation, doses of immunosuppressive drugs, presence or absence of rejection episode, or eventual outcome of the graft. In addition, a series of in vitro experiments disclosed that the alteration in EPM of lymphocytes in the transplant recipients is neither reversible nor caused by humoral factors, immunological or pharmacological, presented in the recipients' sera. Thus, we concluded that decreased EPM of non-T lymphocytes in kidney transplant recipients is due to actual alterations in proportions of subsets of non-T lymphocytes and/or alterations in functional status of existing non-T lymphocyte subsets as a response to kidney transplantation.

In the present study, our effort was concentrated on identifying the subpopulation(s) of lymphocytes which is primarily affected and is shown decreased EPM in these patients. The results of the study delineate that there are distinct differences of EPM among three major subpopulations of normal human peripheral lymphocytes. Furthermore, in dialysis patients and transplant recipients, EPM of both T and B lymphocytes are significantly reduced, while EPM of null cells is unchanged, as compared to EPM of respective subpopulations of lymphocytes in normal subjects. In this study, again, the low EPM of B lymphocytes has been shown uniformly in transplant recipients, regardless of duration and outcome of transplant, doses of immunosuppressive drugs, presence or absence of rejection episode, and degree of graft function.

Unlike the previous study, however, EPM of T-lymphocytes in both hemodialysis patients and transplant recipients, was also significantly reduced from that of normal subjects, but there was no difference in EPM of T-lymphocytes between hemodialysis patients and transplant recipients.

The present study generally supports our previous contention that reduced EPM of peripheral lymphocytes in transplant recipients is a non-specific phenomenon, as discussed in the previous report in detail. Furthermore, measurement of EPM of peripheral lymphocytes from these patients seems to have neither diagnostic (rejection vs. adaptation) nor prognostic (eventual fate of the graft) implications.

As shown in Figures 1-6, although there are considerable differences in mean EPM of lymphocytes between different subpopulations of lymphocytes and different groups of study subjects, major portions of EPM histograms are so closely superimposed, it appears unlikely that one subpopulation of lymphocytes can be completely separated from another by means of lymphocyte electrophoresis alone, even when preparatory electrophoresis is readily available. However, it is possible that if the net electrical charge of a subpopulation of lymphocytes is modified by chemical or immunological treatment, that subpopulation can be separated from the others. Such treatments, both immunological and chemical, can be explored and optimized by further research in lymphocyte surface receptors.

Conclusions

Electrophoretic mobility of peripheral T and B lymphocytes and null cells was determined from normal adults, hemodialysis patients and kidney transplant recipients by using a Cylindrical Microelectrophoresis Apparatus, and the following conclusions have been drawn from this study:

1. Each subpopulation of peripheral lymphocytes has distinct EPM, and T lymphocyte has the highest mobility, while null cell has the lowest mobility.
2. Mean EPM of both T and B lymphocytes from hemodialysis patients and transplant recipients is significantly lower than that of normals.
3. EPM of null cells is identical between normal subjects, hemodialysis patients and transplant recipients.

B. Preparatory Electrophoresis of Human Peripheral Lymphocytes.

To Explore the potentiality of lymphocyte electrophoresis as a means for separation of lymphocyte subpopulations, preparatory electrophoresis of peripheral human lymphocytes was attempted three times with collaboration of Dr. Richard N. Griffin, Space Sciences and Laboratory, General Electric Company, Valley Forge, PA.

The lymphocytes from peripheral blood were prepared as described previously (total approximately 6×10^8 cells with over 85% viability by dye exclusion test).

The following morning, the lymphocytes suspended in Hank's balanced salt solution were brought to Space Sciences and Laboratory of General Electric Co., Valley Forge, in an ice box. When the lymphocytes were suspended in electrophoretic media (phosphate buffered Hanning's solution) for electrophoresis, however, the lymphocytes started to clump together within 15-20 minutes. Thus, a large portion of the cells were lost during this procedure. After the clumps were removed, the cells were resuspended in a new electrophoretic medium [0.01 N NaCl with 4.9% Sorbitol (300 mOsmol) at pH 7.2], and were electrophoresed at a rate of 0.8 ml/hr, with Backman's continuous flow electrophoretic device. Fractionated lymphocytes were collected in 30 tubes according to order of electrophoretic mobilities of lymphocytes. At the end of the procedure, electrophoretic medium was removed from the tubes by centrifuge, and the fractionated lymphocytes were resuspended with Hank's balanced salt solution and brought back to New York.

The next day, a cell count and viability test were performed and the results were as follows:

- i. The total number of lymphocytes recovered after electrophoretic fractionation was 6.24×10^5 , which approximates 1/1000 of the original lymphocytes we had prepared.
- ii. Approximately 95% of the lymphocytes were recovered from the tubes between No. 10 - 15, out of the total 30 collection tubes (Figure 7).
- iii. Viability of the lymphocytes fractionated in the tubes varied from 32% to 72%.

Although the extremely low recovery rate of the lymphocytes might have been due to lymphocyte clumping prior to electrophoresis, both efficiency of fractionation and viability of fractionated lymphocytes in this experiment were also relatively poor.

Since this experiment was only a pilot study and was not conducted under optimum conditions, we attempted two more such experiments. At this time, transportation of lymphocyte specimens between New York and Valley Forge was made by special delivery via air mail. On both occasions, however, the fractionated specimens did not arrive in New York in time and all lymphocytes were disintegrated during the transportation period. Unfortunately, due to the schism of essential facilities in New York and Valley Forge, these and future experiments cannot be conducted successfully.

C. Electrophoretic Mobility of Peripheral Lymphocytes Treated with Immunoglobulin

Since the results of the previous and present studies indicate that it appears unlikely that subpopulations of lymphocytes can be separated by means of the electrophoretic technique alone, we attempted to alter the electrochemical property of the lymphocyte surface by antihuman IgG antibody (immunoglobulin) treatment. We hoped to see that modification of the surface electrical charge of one subpopulation, for example B lymphocytes in this experiment, by such immunologic treatment would augment the difference of the electrophoretic property of that subpopulation from others, so that the treated subpopulation can be electrophoretically separated from the non-treated subpopulation, or visé versa.

Materials and Method

Peripheral lymphocytes from three normal subjects were prepared to enrich T and B lymphocytes as described previously (E rosetting technique for T lymphocyte preparation and EAC rosetting technique for B lymphocyte preparation). Both lymphocyte preparations were suspended in phosphate buffered saline containing either 10% fetal calf serum (Control Group) or rabbit's antihuman IgG antibody (human γ chain specific) at a final concentration of 2.5×10^6 lymphocytes per ml. and 1.25 mg/ml rabbit's IgG (Experimental Group), and were incubated at 4°C for 18 hours. The following day, the treated cell preparations were washed twice with electrophoretic suspending media, and resuspended in electrophoretic suspending media to desired concentration. Chemical composition of the media, the electrophoretic apparatus and the procedure of electrophoresis used in this experiment were described in detail in previous reports.

Results

One uniform finding in this experiment was that both T and B lymphocytes treated with rabbit's antihuman IgG antibody tended to clump together, while the lymphocytes incubated with fetal calf serum remained in suspension. Major portions of the reacted lymphocytes were, thus, probably lost when the clumps were removed from phosphate buffered saline prior to electrophoresis. Therefore, we were able to study EPM of treated lymphocytes from the small

number of those lymphocytes (20 to 30 lymphocytes per case) which did not form clumps but remained in suspension.

The results of EPM of these lymphocytes are shown in Table 2.

Comments and Conclusion

Since we lost a large portion of those lymphocytes, which apparently responded to the rabbit's antihuman IgG antibody treatment, in the Experimental Group, observed EPM of lymphocytes from that group unlikely represent true EPM of the treated lymphocytes. Thus, the present study failed to provide data on the EPM of lymphocytes treated with antihuman IgG antibody. To obtain such information, it is prerequisite that any treatment to modify electrochemical characteristics of the lymphocyte surface should not associate with clumping of the reactive lymphocytes.

Although we have not studied this possibility, we suspect that use of F ab portion only, instead of the entire molecules, of rabbit's antihuman IgG antibody, may alter the lymphocyte surface property without induction of clumping problems. Another possibility is that a short reaction period, such as 1 to 4 hours incubation of lymphocytes with immunoglobulin, may prevent lymphocyte aggregation or clumping, and it is worth trying in the future.

Legend to Figures

- Figure 1. Electrophoretic Mobility of Lymphocytes in Normal Subjects
- Figure 2. Electrophoretic Mobility of Lymphocytes in Hemodialysis Patients
- Figure 3. Electrophoretic Mobility of Lymphocytes in Transplant Recipients
- Figure 4. Electrophoretic Mobility of T Lymphocytes
- Figure 5. Electrophoretic Mobility of B Lymphocytes
- Figure 6. Electrophoretic Mobility of Null Cells
- Figure 7. Fractionation of Peripheral Lymphocytes by Beckman's Continuous Flow Electrophoresis

Legend to Table

- Table 1. Electrophoretic Mobility of Fractionated Peripheral Lymphocytes from Normal Adults, Hemodialysis Patients and Kidney Transplant Recipients
- Table 2. Electrophoretic Mobility of T and B Lymphocytes Treated with Rabbit's Antihuman IgG Antibody

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN NORMAL SUBJECTS

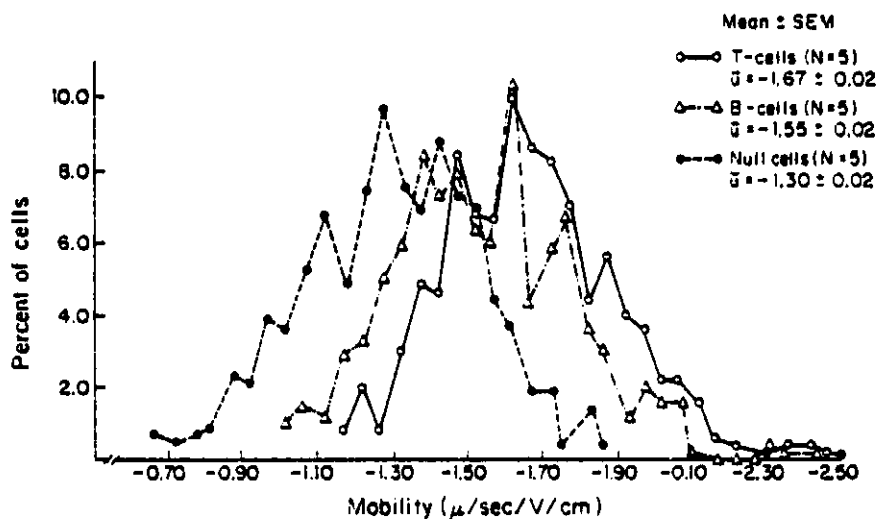


Figure 1

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN HEMODIALYSIS PATIENTS

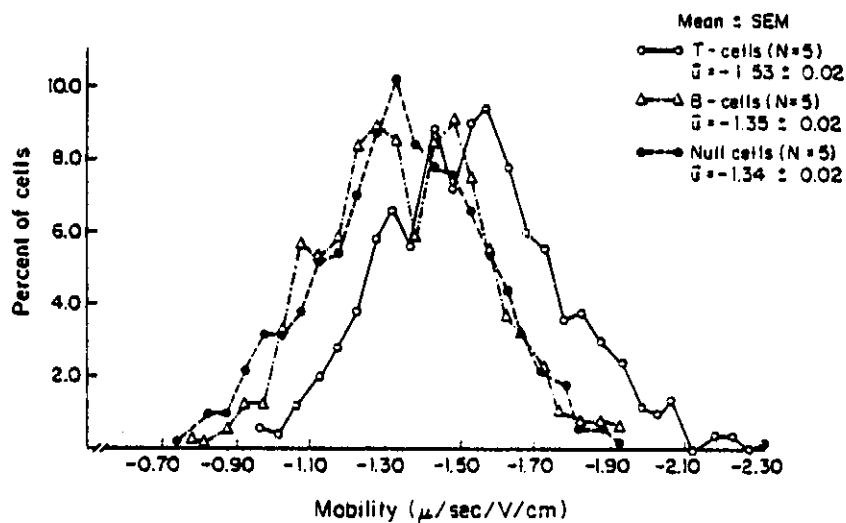


Figure 2

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN TRANSPLANT RECIPIENTS

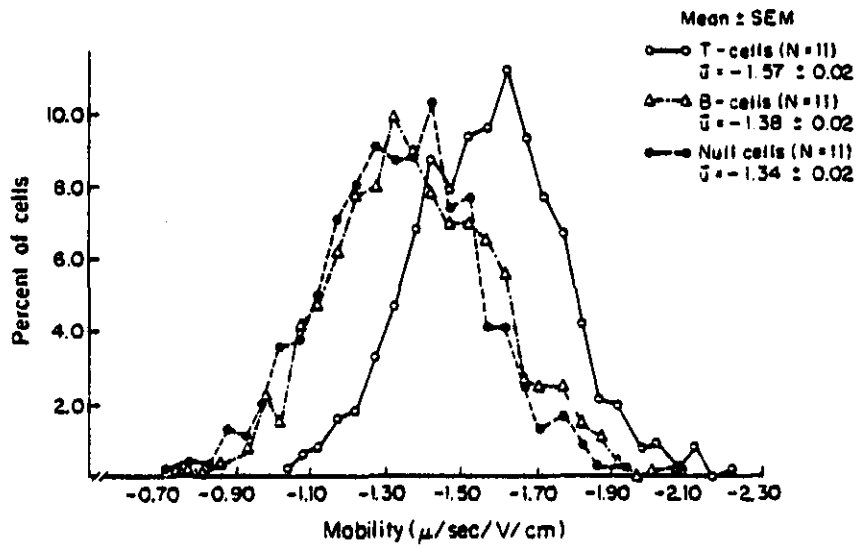


Figure 3

ELECTROPHORETIC MOBILITY OF T LYMPHOCYTES

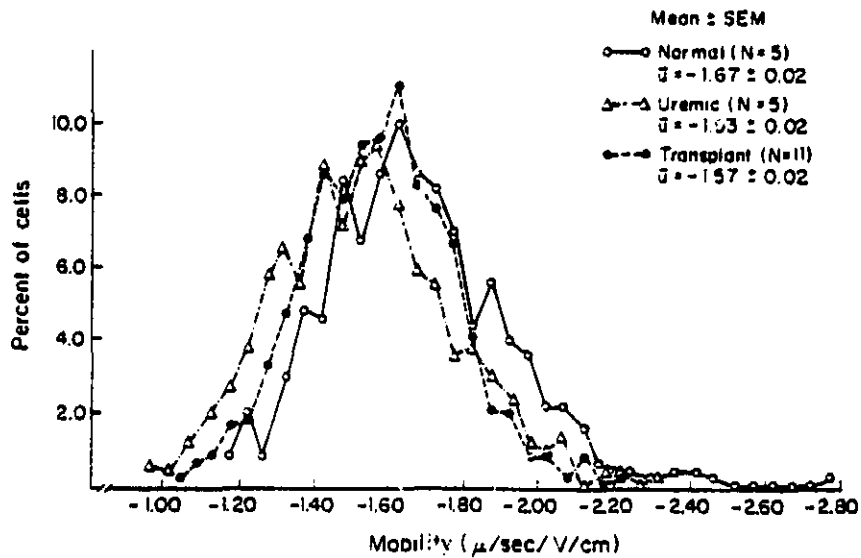


Figure 4

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ELECTROPHORETIC MOBILITY OF B LYMPHOCYTES

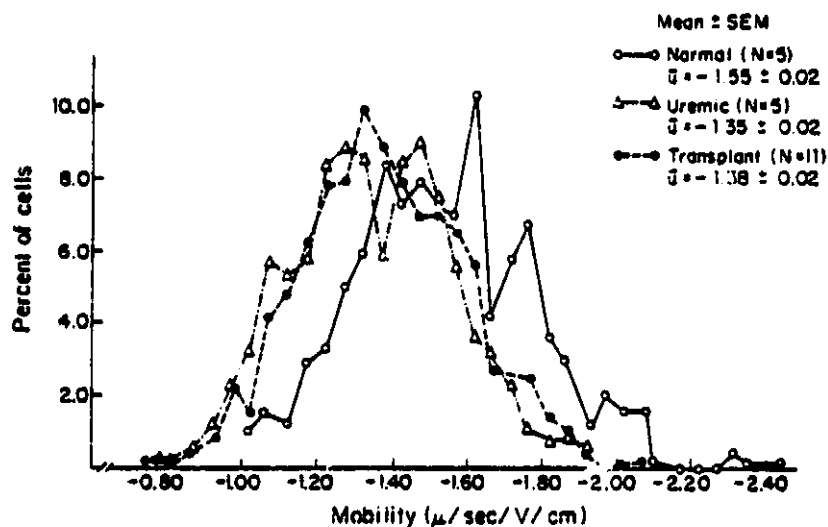


Figure 5

ELECTROPHORETIC MOBILITY OF NULL CELLS

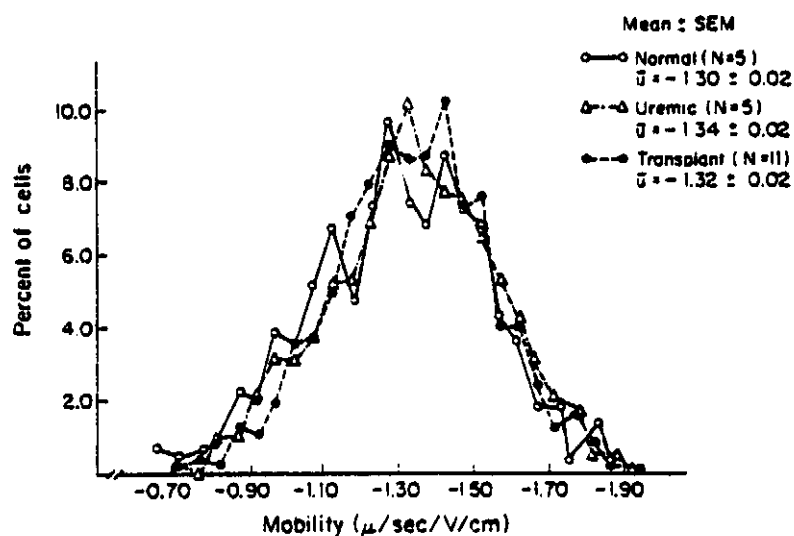


Figure 6

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Figure 7. Fractionation of Peripheral Lymphocytes
by Beckman's Continuous Flow Electrophoresis.

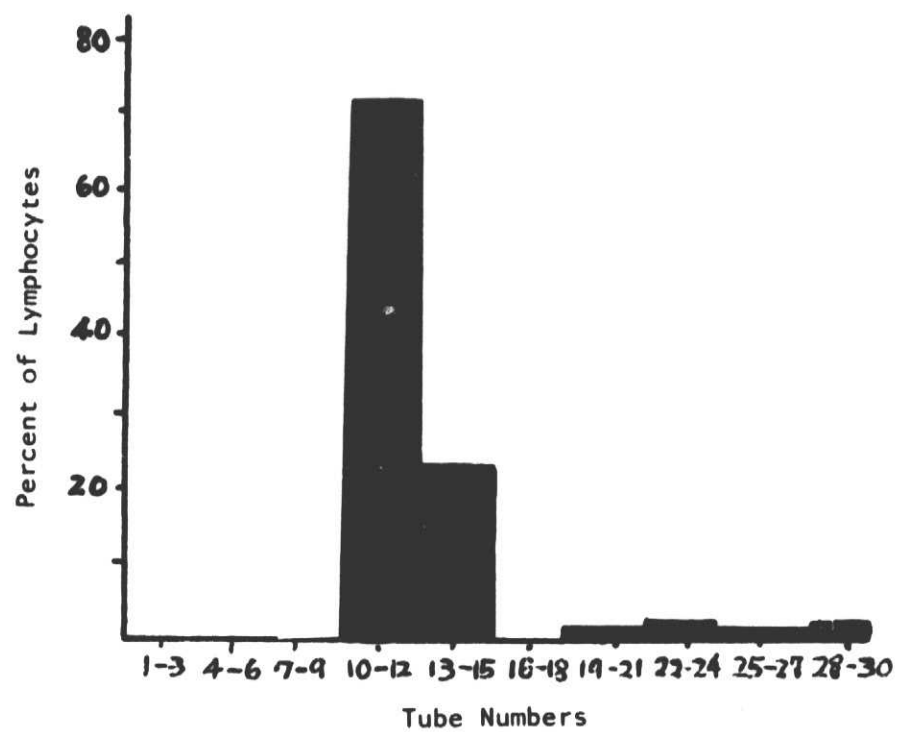


Table 1. Electrophoretic Mobility of Fractionated Peripheral Lymphocytes from Normal Adults, Hemodialysis Patients and Kidney Transplant Recipients

	ELECTROPHORETIC MOBILITY (μ /sec/V/cm)					
	T Lymphocytes		B Lymphocytes		Null Cells	
	<u>Range</u>	<u>Mean $\pm$ SE</u>	<u>Range</u>	<u>Mean $\pm$ SE</u>	<u>Range</u>	<u>Mean $\pm$ SE</u>
Normal (N = 5)	-1.15 - -2.49	-1.67 $\pm$ 0.02	-1.00 - -2.44	-1.55 $\pm$ 0.02	-0.65 - -1.84	-1.30 $\pm$ 0.02
Hemodialysis Patients (N = 5)	-1.00 - -2.29	-1.53 $\pm$ 0.02	-0.75 - -1.94	-1.35 $\pm$ 0.02	-0.70 - -1.94	-1.34 $\pm$ 0.02
Transplant Recipients (N = 11)	-1.00 - -2.24	-1.57 $\pm$ 0.02	-0.70 - -2.09	-1.38 $\pm$ 0.02	-0.70 - -1.94	-1.34 $\pm$ 0.02

Table 2. Electrophoretic Mobility of T and B Lymphocytes Treated with Rabbit's Antihuman IgG Antibody.

<u>Case No.</u>	<u>Electrophoretic Mobility</u> (u/sec/V/Cm) Mean $\pm$ SD			
	<u>Control</u>		<u>Immunoglobulin Treated</u>	
	<u>T</u>	<u>B</u>	<u>T</u>	<u>B</u>
Case #1	-1.70 $\pm$ 0.17	-1.69 $\pm$ 0.24	-1.48 $\pm$ 0.21	-1.64 $\pm$ 0.26
Case #2	-1.66 $\pm$ 0.26	-1.71 $\pm$ 0.24	-1.74 $\pm$ 0.20	-1.63 $\pm$ 0.23
Case #3	-1.77 $\pm$ 0.17	-1.72 $\pm$ 0.25	Clumped - No Cell Available	-1.66 $\pm$ 0.19

PART II. STUDIES ON LYMPHOCYTE ACTIVATION

Our studies on lymphocyte cell-surface phenomenon have been continued through an examination of the effect of microtubular disruption on lymphocyte activation induced by cell to cell contact.

Drugs that disrupt microtubular assemblies inhibit various aspects of mitogen- and antigen- induced lymphocyte activation¹⁻³. The microtubular apparatus modulates cell shape and surface architecture⁴⁻⁵ and has also been implicated in mediating stimulatory signals from the plasma membrane to intracellular sites¹. Thus, disruption of microtubules could affect lymphocyte activation by two distinct mechanisms: (1) by affecting surface interactions between membrane sites and stimulatory agents (i.e., mitogens, antigens, and allogenic cells); and (2) by interfering with propagation of stimulatory signals. Colchicine, a drug that binds to and disrupts microtubules, inhibits mitogen-induced ³H-thymidine (³H-TdR) incorporation¹⁻³, but has no effect on some of the early changes associated with lymphocyte activation². A recent report noted that the stimulation of neuraminidase-treated lymphocytes by galactose oxidase is not inhibited by colchicine at concentrations that inhibit the mitogenic response induced by concanavalin A (Con A)⁶. We report here that colchicine potentiates the mitogenic effect of sodium periodate (IO_4^-), a mitogen that requires cell-cell interaction to induce blastogenesis⁷⁻⁸. This potentiation is obtained by brief incubation of cells with colchicine followed by washing to remove excess reagent. Under these experimental conditions, the mitogenic effect of Con A is inhibited.

Figure 1A and 1B illustrate the effect of pretreating cells with varying concentration of colchicine on ³H-TdR incorporation induced by IO_4^- and Con A. Under these conditions, the response to IO_4^- is progressively enhanced up to a colchicine concentration of 10^{-6} M, and then returns to baseline levels as the colchicine concentration is further increased to 10^{-4} M. The response to Con A, on the other hand, is progressively inhibited by colchicine at 10^{-7} to 10^{-5} M. ³H-Uridine and ³H-leucine incorporation were also used to quantitate the stimulatory effect of IO_4^- and Con A using the experimental conditions outlined in Figure 1. Incorporation of these precursors gave similar results to those for ³H-TdR incorporation. When colchicine was allowed to remain in the cultures (Figure 1C), there was a decrease in ³H-TdR incorporation by cells stimulated with either IO_4^- or Con A.

The potentiation of the mitogenic effect of IO_4^- by colchicine was found both when the cells were pretreated with colchicine followed by IO_4^- and when the cells were first treated with IO_4^- followed by a 30 min incubation with colchicine (Figure 2). It was noted that cells preincubated for 30 min with or without colchicine respond maximally at lower concentrations of IO_4^- than do non-preincubated cells (Figure 2). Nevertheless, the colchicine-induced enhancement of the response is seen at all IO_4^- concentrations tested. These experiments indicated that the enhancing effect of colchicine is not related to exposure of target sites for IO_4^- action.

Lumicolchicine, a photo-inactivated derivative of colchicine, was compared with colchicine for its effect on the mitogenic response to IO_4^- and Con A. Lumicolchicine does not bind nor disrupt microtubules, but shares some other, nonspecific, properties with colchicine⁹. Figure 3 illustrates a typical experiment. Enhancement of IO_4^- and inhibition of Con A responses by colchicine are again noted, but lumicolchicine had little or no effect. These data indicate that the effects of colchicine, seen under our experimental conditions, are related to colchicine binding and disruption of microtubules.

The extent of residual colchicine bound to cells was assayed by determining the amount of ^3H -colchicine bound to extracts (100,000 g X 45 min supernatant)¹⁰ of cells pretreated with unlabelled conchicine (10^{-6}M) for 30 min. 1.03μ moles/mg protein ^3H -colchicine was bound to extracts of cells preincubated without colchicine, and binding decreased to 0.73μ moles/mg protein following preincubation with colchicine.

IO_4^- activates lymphocytes by generating aldehyde moieties on terminal sialyl residues on the cell surface¹¹. IO_4^- modified cells can undergo blastogenesis in the presence of macrophages, and unmodified cells can be stimulated by IO_4^- - treated irradiated macrophages or by IO_4^- - treated lymphocytes in the presence of macrophages⁷⁻⁸. We therefore determined the effect of colchicine on cultures where IO_4^- - treated and irradiated cells are used to stimulate cells that are not treated with IO_4^- . The results (Figure 4) show that enhanced proliferation occurred only when IO_4^- - modified cells (used either as stimulatory or responding cells) were treated with colchicine. The extent of colchicine enhancement of the response of cells treated directly with IO_4^- was greater than that seen by treating IO_4^- - modified and irradiated cells with colchicine and using them to stimulate untreated cells. These findings suggest that the major effect of colchicine is on IO_4^- - modified responding cells.

Blastogenesis induced by IO_4^- requires macrophages for maximal effect¹². Untreated macrophages reconstitute the response of purified T cells to IO_4^- and also reconstitute the enhanced response of colchicine-treated purified T cells to IO_4^- . Thus macrophages are not the target for colchicine action.

These data suggest that colchicine enhances IO_4^- - induced blastogenesis by disrupting microtubules, altering cell-surface architecture, and facilitating cell-cell interactions. The inhibitory effect of colchicine on later events in blastogenesis was probably eliminated in our experiments by the brief exposure of cells to colchicine. Preliminary data from our laboratory indicate that colchicine also potentiates proliferation in mixed lymphocyte culture when either stimulating or responding cells are pretreated with the reagent.

Several investigators have reported that cytochalasin B, a drug that interacts with microfilaments, enhances lymphocyte stimulation in response to Con A and phytohemagglutinin as well as to IO_4^- , but does not enhance the response to antigens¹³⁻¹⁴. In contrast to these effects of cytochalasin B, our studies indicate that brief pretreatment with colchicine enhances lymphocyte responses to IO_4^- and to allogenic cells, but not to Con A. These findings suggest that colchicine has a selective effect on lymphocyte proliferation induced by cell-cell interaction. Agents that influence the integrity of the microtubular assembly may therefore modulate the initiation of immune responses involving cell to cell contact.

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FIGURE LEGENDS

Fig. 1 Effect of colchicine on ^3H -TdR incorporation in lymphocytes stimulated by IO_4^- and Con A. Human peripheral lymphocytes were obtained from healthy normal subjects, age 21 - 45, by Ficoll-Hypaque gradient centrifugation¹⁵. Cells ($10^7/\text{ml}$) suspended in phosphate buffered saline (PBS) were treated with colchicine (Sigma), at concentrations specified in the figure, for 30 min at 37° and washed X 2 in PBS. Aliquots were then treated with IO_4^- (10^7 cells/ml in PBS were incubated with 2mM NaIO_4 for 30 min at 0° and washed X 2) and suspended (10^6 cell/ml) in RPMI 1640, containing 5% heat-inactivated fetal calf serum, and incubated (0.2 ml aliquots) in flat-bottom microwells (Microtest II, Falcon 3040) at 37° in a 95% air - 5% CO_2 incubator for 72 hrs¹¹. Additional samples were cultured with or without Con A (2 $\mu\text{g}/\text{ml}$). Cultures were pulsed with 2 μCi ^3H -TdR for the last 20 hr of culture and incorporation into DNA measured¹². Panel A indicates the relative change in ^3H -TdR incorporation (percent incorporation of colchicine-free cultures) of IO_4^- (●) and Con A (○) treated cells after 30 min exposure to colchicine at different concentrations. Points indicate mean $\pm$ SEM of results obtained using 6 - 12 different individuals' cells. Panel B indicates the actual change in ^3H -TdR incorporation (cpm) that results from colchicine treatment of IO_4^- - stimulated cells. ^3H -TdR incorporation into cells stimulated with Con A without preincubation with colchicine was $165 \pm 23 \text{ cpm} \times 10^{-3}$. Panel C shows a typical experiment on the effect of culturing cells with colchicine present throughout the incubation period (IO_4^- - treated cells [●]; Con A - treated cells [○]).

Fig. 2 Colchicine effect IO_4^- - induced lymphocyte proliferation: effect of sequence of treatment. Cells were either pretreated with colchicine followed by IO_4^- (Panel A) or with IO_4^- followed by colchicine (Panel B). ○ = cells exposed to colchicine (10^{-6} M); ● = control cells.

Fig. 3 Lumicolchicine effect on IO_4^- and Con A induced lymphocyte proliferation. Cells were preincubated with either colchicine or lumicolchicine at various concentrations as described in Figure 1 and stimulated with IO_4^- or Con A. Lumicolchicine was prepared as previously described⁹. ● = IO_4^- stimulation of colchicine-treated cells; ○ = IO_4^- stimulation of lumicolchicine-treated cells; ■ = Con A stimulation of colchicine-treated cells; and □ = Con A stimulation of lumicolchicine-treated cells.

Fig. 4 Enhancement of IO_4^- - induced lymphocyte proliferation when either responding or stimulating cells are preincubated with colchicine. Irradiated cells (3,000 R, Cesium source) (S) were treated with IO_4^- ($\text{S}^{\text{IO}_4^-}$) and colchicine ($\text{S}_c^{\text{IO}_4^-}$) and used to stimulate nonirradiated cells, with (R_c) and without (R) colchicine pretreatment. Lymphocytes were also treated directly with IO_4^- ($\text{L}^{\text{IO}_4^-}$) and colchicine ($\text{L}_c^{\text{IO}_4^-}$). Results are expressed as mean $\pm$ SEM (n = 3 individuals' cells) of the percent stimulation of control cells that had not been exposed to colchicine. ^3H -TdR incorporation in the control cultures was $\text{L}^{\text{IO}_4^-} = 40.6 \pm 4.1 \text{ cpm} \times 10^{-3}$; $\text{S}^{\text{IO}_4^-} \text{ R} = 21.1 \pm 8.2 \text{ cpm} \times 10^{-3}$.

FIGURE 1

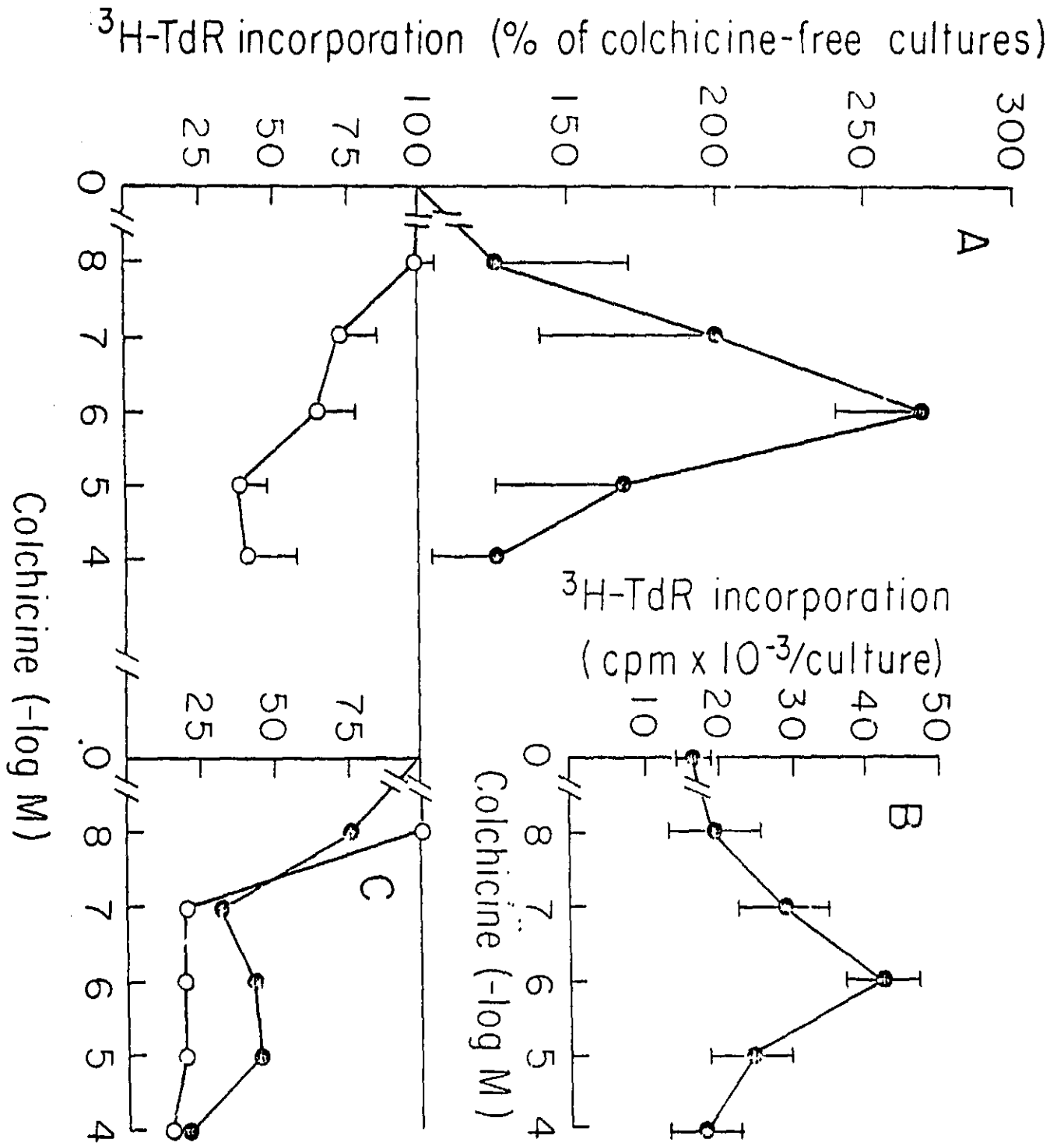


FIGURE 2

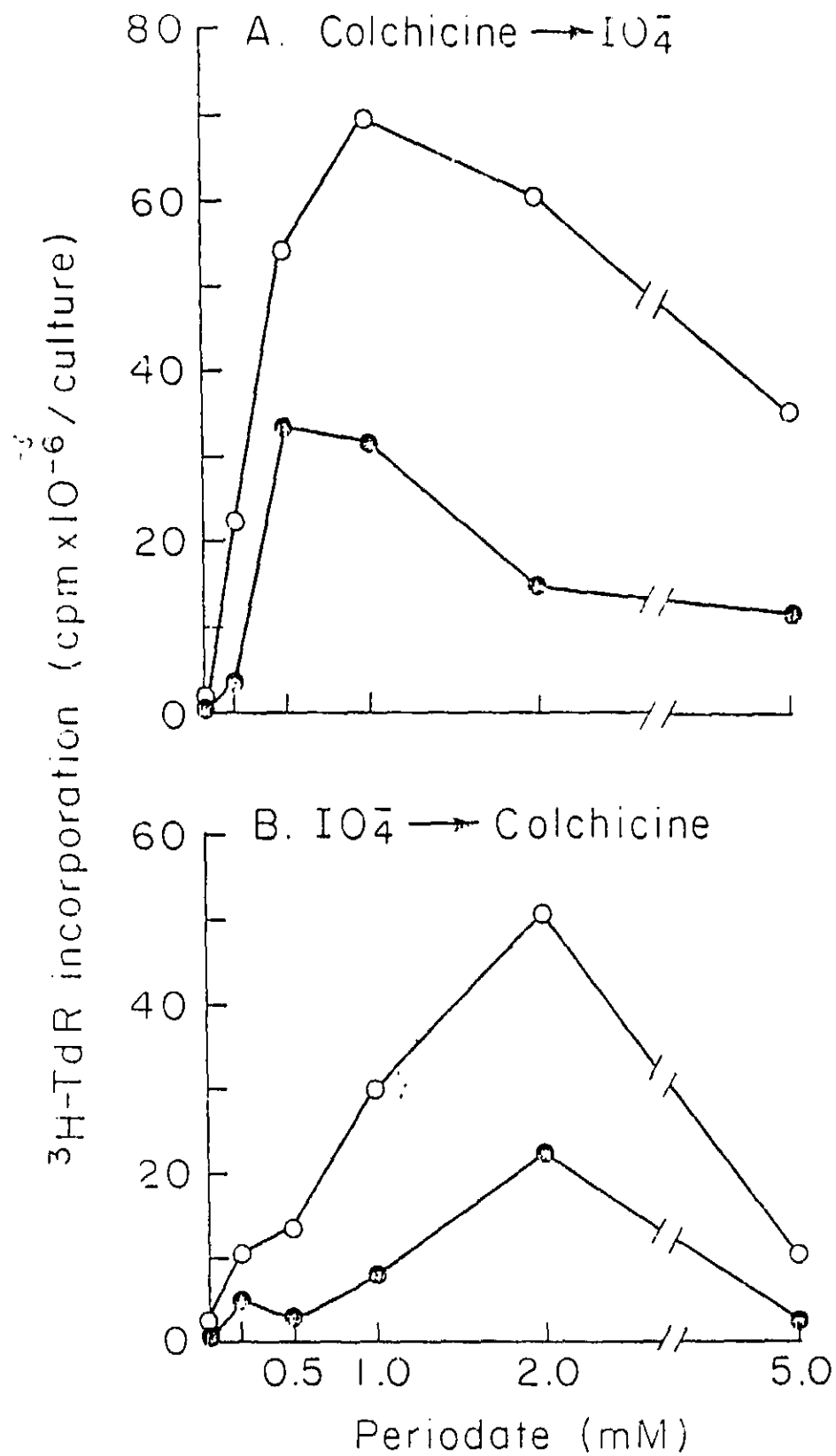


FIGURE 3

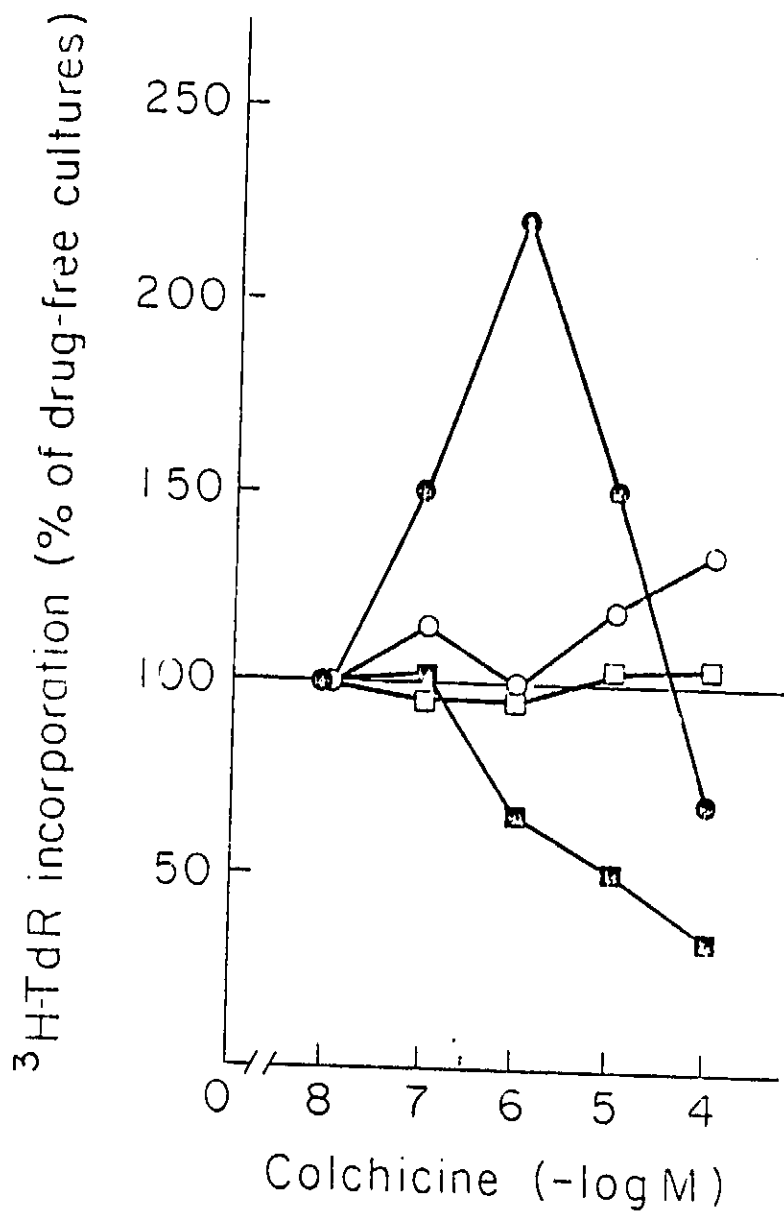
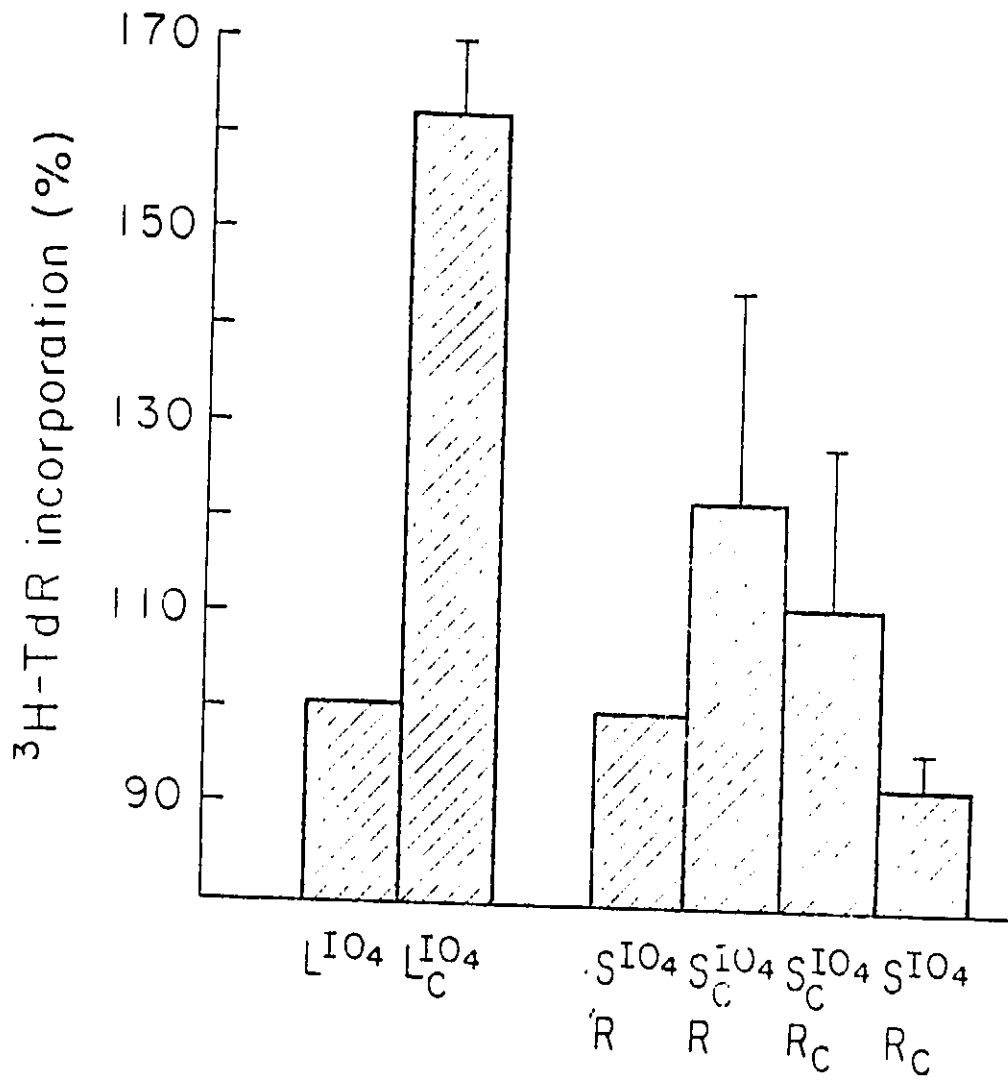


FIGURE 4



SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

These studies have been devoted to an examination of the separation of human peripheral lymphocytes by electrophoresis. A microelectrophoretic technique was utilized to determine the feasibility and properties of this type of separatory procedure with a view toward possible utilization of of preparative lymphocyte electrophoresis at 0 G. The ultimate goals of this research were to separate subgroups of lymphocytes and study their properties with a view toward the eventual use of these subfractions in clinical medicine. Multiple studies utilizing microelectrophoresis have been carried out in both normal individuals and those receiving renal transplants. The transplant recipient group was chosen because they had a well-defined antigenic stimulus. Histograms of the electrophoretic mobility of human lymphocytes and lymphocyte subpopulations were analyzed by a variety of statistical methods. The major findings were that human T and B cell lymphocyte subpopulations had a slight difference in electrophoretic mobility; the group of transplant recipients had more polydisperse histograms than the normal group and, when isolated subpopulations were studied separately, it was found that the B cell subpopulations of the transplant recipients had a significantly altered electrophoretic mobility. These studies suggest a change in lymphocyte subpopulations following a profound antigenic stimulus and immunosuppression as occurs during clinical renal transplantation. The techniques of microelectrophoresis are suitable for assessment of the cell's electrophoretic properties, but at their present state of development are not suitable for separation of cells in sufficient quantity for in vitro or in vivo testing.

It became clear from these studies that, since an abnormality in cell populations did occur, it was of paramount importance to develop in vitro test systems that could determine unique functions of different lymphocyte subpopulations. Many of the biologically important immunologic reactions involving B as well as T cells are mediated by cell-cell interactions. One of the immediate problems that we recognized was the lack of a suitable system for analyzing cell-cell interactions and the role lymphocyte subpopulations might have in these interactions.

Therefore, we propose to continue this research by developing a model system for the study of lymphocyte activation via cell-cell interactions.

Previous studies in our laboratories have shown that agents that generate aldehyde groups on the surface of lymphocytes lead to extensive blastogenesis. Studies utilizing this system indicate that the induction of blastogenesis by sodium periodate or galactose oxidase treatment of the cells, treatments that lead to the generation of aldehyde groups on the cell surface, require several lymphocyte subpopulations, and involve different types of cell-cell interactions. Aldehyde-modified cells can stimulate unmodified cells, provided that a third cell subpopulation, i.e., macrophages, are present in the lymphocyte culture. In addition, aldehyde-modified cells themselves may proliferate, provided again that macrophages are present.

Thus, this system is composed of three interacting cellular elements: [1] the stimulatory cell--that is, the aldehyde-modified cell (S); [2] the responding cell (R); and [3] the helper cell, or macrophage (H).

We propose the following studies to define and characterize this model-system for the study of lymphocyte activation induced by cell-cell interaction:

1. General Characterization of the System

- (a) Assessment of the quantitative requirement for each cell type (S, R, and H cells).
- (b) The effect of various doses of the aldehyde-generating agents (periodate or galactose oxidase) on the function of S and R cells.
- (c) A determination of the time course of the blastogenic response in the system under various experimental conditions, especially when the stimulatory cell or the responding cell is modified.

2. Nature of the Cells That Compose This Triple-Cell Model System

(a) The Nature of R Cells. Previous studies have shown that the responding cell in this experimental system is a T cell. However, it is known that the T cell subpopulation is heterogeneous, and this was also found in our studies on electrophoretic mobility of lymphocytes. These studies demonstrate a polydispersity of the T cell subpopulation. As an extension of the studies on the electrophoretic mobility of T cells, we have now developed methods for quantitative separation of two of these T cell subgroups (T_y and T_μ). This separation is based on the nature of their surface receptor for the Fc portion of either IgG or IgM.

(b) The Nature of S Cells. Previous studies have indicate that aldehyde modification of unfractionated human peripheral lymphocytes render them capable of functioning as stimulatory cells. However, the ability of purified T subgroups to function as S cells following aldehyde modification should be evaluated. In addition, it would be of the utmost interest to study the role of B cells as S cells in this system. In this connection it would be of particular interest to study the properties of B cells from transplant recipients, since a marked abnormality in electrophoretic mobility was found in this group of cells.

(c) The Nature of H Cells. Previous data indicate that cells that adhere to glass or plastic surfaces can function as helper cells in this system. Macrophages or monocytes can function both as helper and stimulatory cells. The function of B cells and adherent T cell subpopulations as helper cells must also be investigated. It would also be of interest to determine the function of non-lymphoid cells as helper cells in this system, i.e., fibroblasts, endothelial cells, or other human cell types.

3. Nature of the Stimulatory Site Generated by Periodate and Galactose Oxidase

This system provides a means to study the chemical nature of a site that is involved in activating lymphocytes via cell-cell interactions. We will examine the two possible ways by which this site could be generated. The stie may be identical to the aldehyde group generated on the cell surface, or it may be related to other membrane sites that are generated as a consequence of the surface alteration by the aldehyde-generating reagents. This problem will be approached by the use of reagents known to react with specific sties on the cell surface, such as aldehyde-blocking agents and immunoglobulins directed against products of the major histocompatibility locus. In addition, we plan to analyze membrane components (as revealed by polyacrylamide gel electrophoresis) of cells that are capable of functioning as S cells. The metabolic requirements for the generation of the stimulatory site will also be investigated.

Finally, since the reactions discussed above depend on cell-cell interactions, they may be profoundly affected by 0 gravity conditions. This system might, then, provide a unique experimental model to study the effects of 0 gravity on some aspects of the immune response, especially those intimately associated with cell-cell interactions.