Early Detection of Disease Program: Evaluation of the Cellular Immune Response

FINAL REPORT

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Early Detection of Disease Program:

Evaluation of the Cellular Immune Response

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I. Introduction

The objective of the proposed study as stated in the Statement of Work was to evaluate the early cellular responses of specific components of the leukocyte and epithelial cell populations to foreign challenges of both an infectious and noninfectious character. Procedures for screening potential flight crews were to be developed, documented, and tested on a control population.

These goals have been met with the exception that a larger population of people needs to be studied for adequate statistical reliability.

The first year was devoted to establishing and evaluating methods for preparing suitable populations of lymphocytes, polymorphonuclear leukocytes, macrophages, and epithelial cells. Lymphocytes were studied for normal function, and baseline normal values were determined during this period. Epithelial cells from viral infected individuals were screened with a number of anti-viral antisera now available in our laboratory. This procedure showed the earliest indication of disease as well as providing a specific diagnosis to the physicians. A complete conventional virus diagnostic laboratory was and is now operational with diagnostic samples done on physician request. Both macrophages and polymorphonuclear leukocytes were studied from normal individuals, smokers, and patients with viral infections.

The second year has seen the development of newer techniques enabling better definition of lymphocyte subpopulations, namely the E and EAC rosette procedures for recognition of T (thymus-derived) and B (bone-marrow-derived) lymphocyte subpopulations. Lymphocyte and lymphocyte subpopulation response to multiple mitogens have been evaluated. The effect of storage on whole blood before processing was determined. An additional 160 blood samples were added to the normal baseline values for our laboratory. Studies to determine reproducibility of normal data were performed. Many of these baseline values were obtained in conjunction with studies of a Rhinovirus, three influenza (England II and Hong Kong) viral infections and Mycoplasma respiratory infection. Lymphocyte analyses by microspectrophotometry were expanded to correlate morphological changes during blastoid transformation with nucleic acid/protein kinetics. Baseline microspectrophotometric values were obtained on lymphocytes from both normal and immunodeficient individuals. Lymphocyte response to non-specific mitogenic challenge was evaluated.

II. Overall Progress toward Goals

The objectives as defined in the statement of work have been met and the protocol that is recommended in this report has been evaluated on a basis of cellular response in both normal individuals as well as subjects with infectious disease. The recommendations for use of the outlined protocol for early detection of disease must be qualified with the statement that none of the individuals extensively studied constitutes the astronaut population, a factor that may be quite pertinent as far as normal baselines are concerned. Astronauts have consistently shown different clinical values throughout the Apollo and Skylab series. What may be normal to a man on the street may not be a normal value for an astronaut. We do believe that there is no basis to assume that astronauts would respond differently if infected with a viral agent. We recommend that additional studies be done to increase the numbers of individuals in both the control as well as infected study groups and to evaluate the normal values for the astronaut populations.

III. Completed work on sectional phases

A. Lymphocytes

1. Establishment of Baseline Values, Procedures and Statistical Variation in Non-Ill Individuals:

Introduction

The cellular immune system is comprised of thymic derived (T) lymphocytes and bone marrow derived (B) lymphocytes (Good, 1970; and Raff, 1971). The T cells are identified by their capacity to form rosettes with sheep erythrocytes (Jondal, Holm, and Wigzel, 1972), their lack of surface bound immunoglobulin (Raff, 1970; Crane, Kock, and Simonsen, 1972) and their responsiveness to Concanavalin A and phytohemagglutinin (PHA) mitogens (Douglas et al., 1969; Andersson et al., 1972). They appear to be responsible for cell-mediated immunity.

B lymphocytes have surface membrane bound immunoglobulin detectable by immunofluorescence (Raff, 1971), have C'3 complement receptors (Bianco et al., 1970), and are mediators of humoral immunity.

The present study was conducted to define the normal reproducible ranges of B and T lymphocytes in 160 non-ill individuals. To determine the variability of the sampling procedure, multiple tubes of blood were drawn on one individual and values compared. Test reproducibility was determined by a blind study of multiple counts on one sample, and multiple samples from one individual. Variation

among individual people was studied over a 3-13 week period. Additionally, studies were done to determine how long blood samples could be stored and at what temperatures before changes were noted.

MATERIALS AND METHODS

Selection of subjects.

One hundred-sixty subjects were selected from healthy prison volunteers, medical students and Baylor laboratory personnel.

Blood Collection.

Samples were collected in commercially available heparinized vacutainer tubes (#4716 Becton-Dickinson, Rutherford, N.J. 07070), or tubes to which 200 units sterile preservative free heparin (Fisher Scientific Co., Houston, Tx.) had been added for each 10 ml blood. The samples were processed within 1 hr after collection with the exception of some that were held at $4^{o}C$ or room temperature for 24 , 48 or 72 hrs before processing.

White blood cell counts and differentials.

WBC counts were performed using the Unopette diluting system (#5855, B-D) and hemacytometry. Some counts were run on a Coulter Counter (Model Fn, Coulter Electronics, Hialeah, Fla.) parallel with the manual method. Slides for differentials were prepared with the standard technique and stained using the Ames Hematek Slide stainer (Ames Corporation, Elkhart, Indiana).

ABO Blood group typing.

The ABO blood group of each sample was determined using commercially available antisera (Dade, Miami, Fla.) by the slide agglutination technique.

Lymphocyte separation.

Purified lymphocyte suspensions were obtained using the Lymphocyte Separator ^{TM} (Technicon Corporation, Tarrytown, N.Y. 10591). The whole blood was centrifuged at 2000 RPM for 15 min, and the buffy coat removed. The separating reagent (Technicon) was mixed well, and an amount 4 times the volume of the buffy coat was drawn into a 50 ml plastic disposable syringe using a 20 gauge needle. One ml of the appropriate ABO antiserum and the buffy coat were then added. After drawing an airspace equal to the volume of liquid and plugging the needle with a vinyl cap, the syringe was placed on the Technicon Rotator in a 37^{o}C incubator and allowed to rotate for 30 min. After the rotation period, the airspace was removed, the needle replaced, and the syringe was placed upright in a rack for 30 min at 37^{o}C. Following sedimentation, the sample was processed through the lymphocyte separator. The sample collected was then centrifuged for 10 min at 2000 RPM and the supernatant removed. The cells were washed 3 times with phosphate buffered saline (pH 7.4). The cells were resuspended in PBS, a cell count performed, and the cells then aliquoted into various tubes for testing.

B lymphocytes.

1.0×10^6 lymphocytes were placed in each of 4 tubes. 2-3 drops of a 1:4 dilution of a FITC-conjugated antisera to total anti-human immunoglobulin, anti-IgG, anti-IgA and anti-IgM, were placed in the appropriate tubes. After mixing, the cells were allowed to react with the antiserum at $4^{\circ}C$ for 30 min. They were then washed three times with cold PBS, placed on a glass slide, coverslipped, and examined for the number of fluorescent cells by use of an Ortholux Leitz Microscope equipped with an HBO 200 mercury burner, phloen illuminator, with a S 360 (UG1) UV exciter filter, a K 460 barrier filter, and a S 525 interference filter (fluorescence selection filter).

Rosette forming lymphocytes (T cells)

Sheep RBC (SRBC) were stored at $4^{\circ}C$ in Alsever's solution, washed 3 times with minimum essential media (Eagle) (Gibco) (MEM), and adjusted to a 0.5% suspension. 1.0×10^6 lymphocytes were placed in tubes and washed once with MEM. 0.25 ml MEM and 0.25 ml SRBC suspension were added to each tube. After mixing, the tubes were incubated at $37^{\circ}C$ for 5 min. The cell suspension was centrifuged at 500 g for 3 min., then incubated in ice water for 2 hrs. Approximately half of the supernatant was removed and the top-layer of cells gently resuspended. Both sides of a hemacytometer were filled and 100 lymphocytes were counted on each side. Lymphocytes with 3 or more adhering SRBC were counted as rosette forming cells.

Non-reactive lymphocytes ("null" cells)

Non-reactive cells were determined by adding the immunoglobulin bearing and the rosette forming T lymphocytes and subtracting the value from the total number of lymphocytes.

Culture technique

Lymphocyte cultures were set up by the method of Curtis et al. (1970) with the following exceptions: 1×10^6 lymphocytes were suspended in 1 ml of fetal calf serum (Gibco #614). The cell suspensions were placed in 16 x 125 mm sterile culture tubes containing 2 ml of MEM Pen-Strep solution (10,000 units Penicillin and 10,000 mcg Streptomycin/ml, Gibco #514) and MEM L-glutamine (200 mM, Gibco #503). Duplicate control cultures and cultures with phytohemagglutinin-P (PHA-P, Difco, 0.01 ml/culture) or pokeweed mitogen (PWM, Gibco, 0.05 ml/culture) were incubated in a 5% CO_2 atmosphere at 37°C. PHA-P cultures were harvested at 3 days and PWM at 5 days. At the time of harvest, 1 µc of ^{3}H-thymidine was added to each tube and incubated for 2 hours at 37°C. The cells were washed with cold saline, the protein precipitated with 5% trichloracetic acid and methanol, solubilized with Soluene (Packard Instruments) and suspended in a toluene scintillation fluid. Activity was measured in a Packard Tri Carb Liquid Scintillation Spectrometer (Model 574) and results expressed as counts per minute (CPM) per culture.

RESULTS AND DISCUSSION

Normal values for 160 non-ill subjects.

Table 1 shows the mean values, standard deviation, and ranges for non-ill subjects studied. The actual number (N) in each test is shown. The values are expressed as absolute numbers of cells per milliliter of whole blood and as percentages. These many parameters will form the data base for comparison with known abnormal subjects in the future.

Technical variation for each test.

Table 2 shows the variation when duplicate samples were drawn from three individuals and each of the duplicate samples processed separately. The total white cell and absolute lymphocyte counts varied by $\pm$ 10%, the T cell-rosette by $\pm$ 5% and B lymphocytes by $\pm$ 10%. In a separate study (not in table), 6 tubes of blood were drawn from one individual to determine the variation due to sample collection. The total leukocyte counts and absolute lymphocyte counts varied by $\pm$ 10%, the rosetting T lymphocytes by $\pm$ 7% and the fluorescent B cells by $\pm$ 5%.

Individual variation

Serial studies showed that variation can occur for an individual over a period of time (Table 3). Different individuals showed a 5 to 20% variation in WBC (but always in the normal range) as did the absolute lymphocyte changes. The T cell-rosette test varied from 5-15% while the total B cells varied from 5-35%. Individual

I from 1-4%

Table 1

Peripheral White Blood Cell Values for Non-ill Individuals

Parameter	n	mean	±1 S.D.
WBC/mm^3	140	6,997	2,370
Segs - %	124	51	11
Lymphs - %	131	45	13
Lymphs - x 10^6/ml	131	3.12	1.13
Total B cells			
- %	160	16	7
- x 10^5/ml	137	4.94	2.97
IgG cells - %	152	8	5
- x 10^5/ml	130	2.47	1.86
IgA cells - %	152	4	3
- x 10^5/ml	130	1.25	1.02
IgM cells - %	152	4	3
- x 10^5/ml	130	1.28	1.02
Rosette forming cells			
- %	95	56	11
- x 10^6/ml	95	1.73	0.64
Non-reactive cells			
- %	93	29	12
- x 10^6/ml	93	0.95	0.54

Table 2

Reproducibility of duplicate samples

Sample	WBC mm^3	Lymphocytes %	Lymphocytes x 10^6/ml	Total B cells x 10^5/ml	IgG cells x 10^5/ml	IgA cells x 10^5/ml	IgM cells x 10^5 ml	Rosette forming cells x 10^6/ml
AA	5,748±443	47±8	2.66±0.37	3.84±0.13	2.86±0.43	0.39±0.16*	0.53±0.04*	1.65±0.34
BB	7,765±651	30±8	2.34±0.77	3.94±0.70	2.97±0.58	1.03±0.09*	0.24±0.06*	1.22±0.46
CC	8,553±487	42±8	3.55±0.75	9.05±0.07	3.99±0.24	2.30±0.17*	2.13±0.07*	2.50±0.51

*SD done with only 2 samples, all others 4 samples.

Table 3

Serial variation of normals over a time period

Sample	Time of Study Wks.	WBC/mm^3	Lymphocytes %	Lymphocytes x 10^6/ml	Total B Cells x 10^5/ml	IgG Cells x 10^5 ml	IgA Cells x 10^5/ml	IgM Cells x 10^5/ml	Rosette forming cells x 10^6/ml
S.C.	13	5,825±580	32±6	1.86±0.30	1.62±0.88	0.85±0.53	0.28±0.26	0.42±0.53	1.07±0.28
D.W.	13	5,971±1,342	38±8	2.27±0.70	2.92±1.78	1.83±1.86	0.76±0.38	0.80±0.51	1.58±0.53
E.B.	7	5,885±1,299	45±17	2.60±0.93	3.63±2.18	1.22±0.85	1.18±0.65	1.85±0.93	1.77±0.53
V.B.	4	5,647±986	41±4	2.35±0.59	3.44±0.87	2.05±0.73	0.84±0.45	0.57±0.24	1.44±0.31
W.L.	4	4,547±446	40±9	1.81±0.39	1.59±0.43	0.58±0.21	0.20±0.20	0.78±0.22	1.21±0.37
D.L.	4	6,277±756	30±3	1.88±0.22	3.57±1.34	1.53±0.86	0.91±0.36	1.16±0.61	1.12±0.26
G.C.	4	4,923±631	33±8	1.59±0.40	1.36±0.26	0.47±0.13	0.19±0.06	0.71±0.32	1.09±0.37
E.G.	4	5,555±900	35±3	1.90±0.21	3.66±2.05	1.82±1.79	0.95±0.52	0.90±0.22	0.91±0.48
W.A.	4	6,078±352	32±13	1.90±0.66	1.72±1.07	0.56±0.23	0.47±0.43	1.06±0.35	0.97±0.52
W.P.	4	6,417±543	38±5	2.44±0.33	2.36±1.12	1.39±0.84	0.33±0.40	0.65±0.14	1.69±0.31
C.L.	3	7,673±942	41±1	3.15±0.41	3.27±1.52	1.82±0.70	0.32±0.35	1.09±0.62	2.04±0.08
N.F.	3	5,243±796	43±6	2.26±0.52	3.00±0.30	1.98±0.21	0.46±0.40	0.56±0.48	1.49±0.21

Effects of storage of whole blood.

Storage studies showed that it was possible to hold blood samples for up to 48 hrs at 4°C without appreciably affecting the results. Table 4 shows the findings. WBC decreased up to 27%, but lymphocyte counts were within 10% of the value of the fresh samples. Morphological studies showed individual variation in the degree of red cell crenation and rupture of polymorphonuclear leukocytes, but rare lymphocyte damage. Lymphocyte viability maintained 88-96% over the 48 hr period; however, in the separated lymphocyte suspensions, the percentage of contamination by polymorphonuclear leukocytes increased as the storage time was prolonged. The rosetting T lymphocytes or the surface bearing immunoglobulin B lymphocytes remained constant during the 48 hr period. At 72 hrs all samples' values had begun to decrease.

Lymphocytes that were stored were cultured with PHA-P and PWM at 0, 24, and 48 hrs (Table 5). The tubes of whole blood were held either at 4°C, 25°C or 37°C as shown in Table 5. Blood held at 4°C showed increasing stimulation indexes for PHA-P and PWM throughout the 48 hr period. Blood held at 25° for 24 hr before processing also gave good stimulation. However, whole blood held at 37°C yielded reduced indexes.

Table 4.

Effect of storage of whole blood on lymphocytes

	WBC/mm^3	Lymphocytes x 10^6	Total B Lymphocytes x 10^5	T Rosetted Lymphocytes x 10^6	Lymphocyte yield x 10^6	Lymphocyte purity* %	Lymphocyte viability %
Fresh	7800±3400	2.0±0.3	3.6±1.8	1.10±0.3	9.4±2.2	98±2	96±2
24 hr	5700±2000	2.1±0.7	4.0±2.2	1.16±0.4	7.8±3.5	90±6	90±7
48 hr	6300±2500	1.8±0.8	4.6±2.8	1.16±0.4	6.3±1.3	85±13	88±5

* Purity from other types of leukocytes.

Table 5

Effect of storage on lymphocyte response to phytomitogens

Length of Storage hrs	temp. °C	PHA-P CPM/culture			PWM CPM/culture		
		Stimulated	Unstimulated	S.I.	Stimulated	Unstimulated	S.I.
0	-	11,500	387	30	15,878	992	16
24	4	9,680	197	49	17,192	1,518	11
24	25	2,938	143	21	17,420	280	62
24	37	755	106	7	1,078	275	4
48	4	40,594	661	61	38,152	254	150

In summary, the values derived from this study of normal people will form the basis for comparison with known abnormals to be studied under controlled conditions in subsequent investigations.

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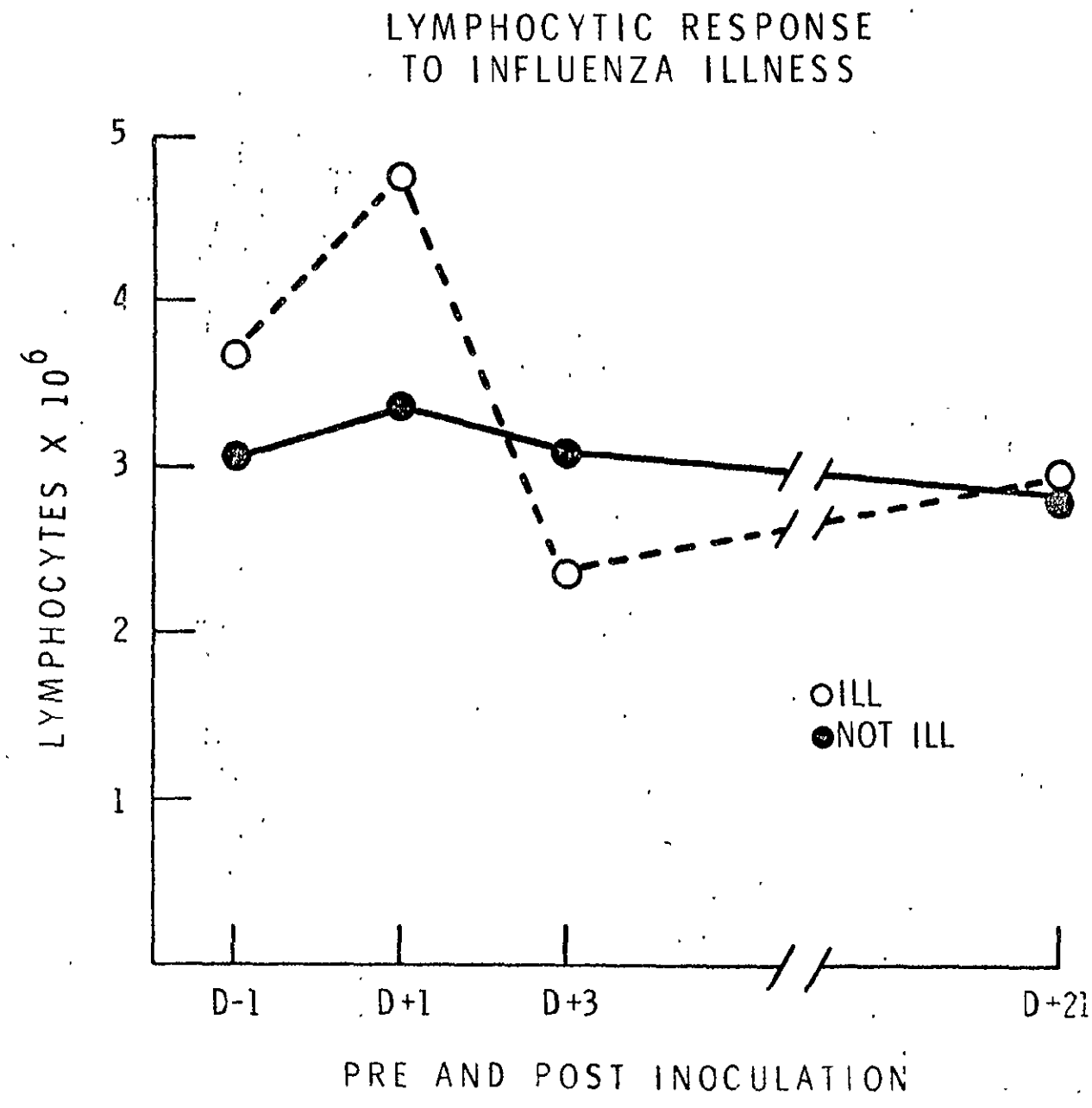
III. A. Lymphocytes (continued).

2. Peripheral Blood Lymphocytic Response to Influenzavirus Infection:

Twenty-two normal individuals were studied serially to determine lymphocyte responses following inoculation with influenza virus. Thirteen individuals showed no clinical symptoms or evidence of disease and were classified as not ill. Nine individuals exhibited symptoms of a flu-like illness and were classified as ill. Previous studies on 160 normal subjects served to establish normal values. White blood cell counts and differentials were done on whole blood, then purified lymphocytes were obtained using the Technicon Lymphocyte Separator. The number of B cells was determined using fluorescein conjugated antisera to total human immunoglobulins, IgG, IgA, and IgM. The sheep red blood cell rosette technique was used to determine the number of T lymphocytes. Cells not reacting to either test were classified as non-reactive lymphocytes. All results were expressed as the number per ml of whole blood.

The absolute lymphocyte counts (Figure 1) on day -1 (day prior to influenza inoculation) for the ill and not ill groups are not different from the normal values (shown previously in section III.A.1.). The not-ill group did not change during the study period. Subjects developing infection and illness, however, showed a significant increase on D+1 compared to normals (4.75×10^6 vs 3.16×10^6/ml,

FIGURE 1


LYMPHOCYTIC RESPONSE
TO INFLUENZA ILLNESS
LYMPHOCYTES X 10^6
5
4
3
2
1
ILL
NOT ILL
D-1
D+1
D+3
D+21
PRE AND POST INOCULATION

$P < 0.005$) followed by a significant decrease on D+3 to 2.37×10^6/ml ($P < 0.025$). By day 21 lymphocyte values returned to normal.

Total B cell levels (Figure 2) were the same for the normals (again Section III.A.1.), the ill, and the not ill groups on D-1. The not ill group did not vary significantly from normal values at any time. However, the ill group showed a highly significant increase ($P < 0.005$) over normal values (10.02 compared to 4.94×10^5) of D+1. These values had returned to normal by D+3 and remained normal at D+21. This increase was due to a 20% increase in IgG cells and a 30% increase in both IgA and IgM cells. Approximately 10-15% of the increase was presumably due to the remaining immunoglobulins (IgD and IgE).

Rosette forming lymphocytes (Figure 3) from ill individuals significantly decreased (1.95 to 1.23×10^6, $P < 0.05$) from their pre-inoculation values on D+3. By D+21, their values were increased as compared to D+3.

The non-reactive lymphocytes (Figure 4) of the not-ill group showed no change from normal values (Section III.A.1.) during the study. The group who got ill showed a highly significant ($P < 0.005$) increase at D+1 as compared to normal values (1.94×10^6 to 1.04×10^6). This was followed by a significant

FIGURE 2
B LYMPHOCYTE RESPONSE
TO INFLUENZA ILLNESS

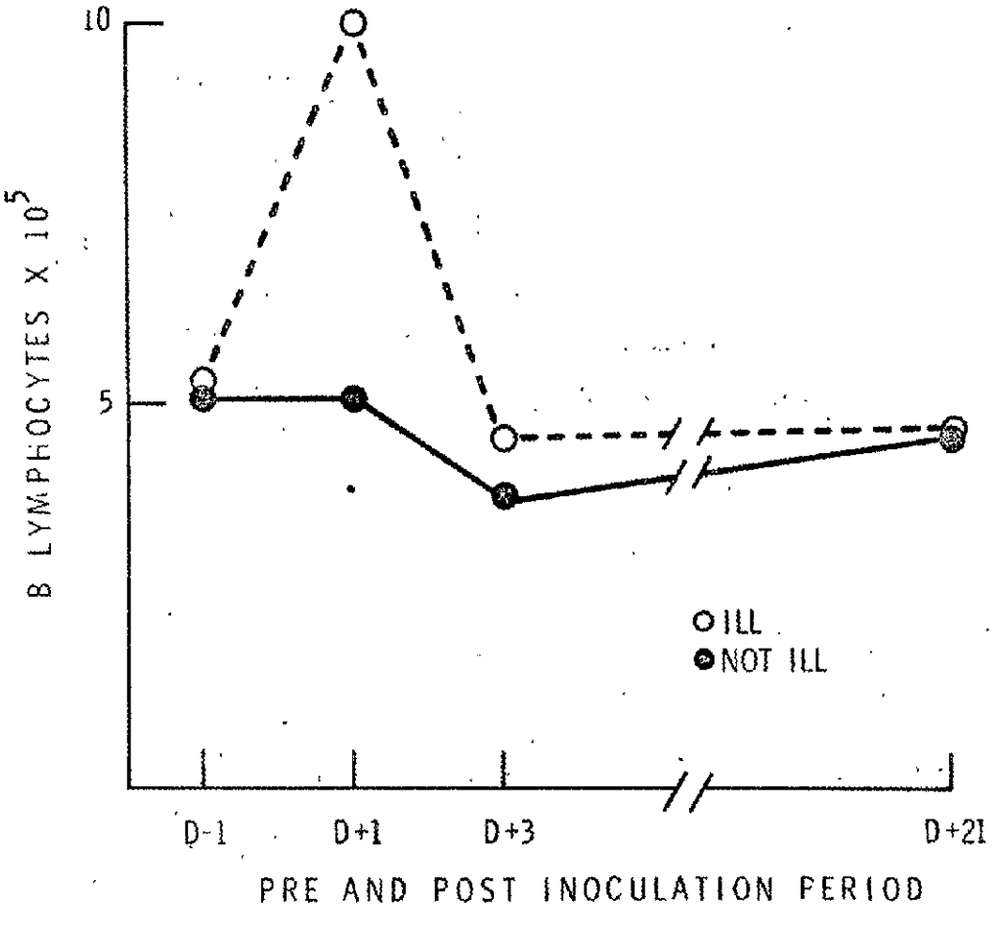


FIGURE 3

ROSETTING T LYMPHOCYTE RESPONSE
TO INFLUENZA ILLNESS

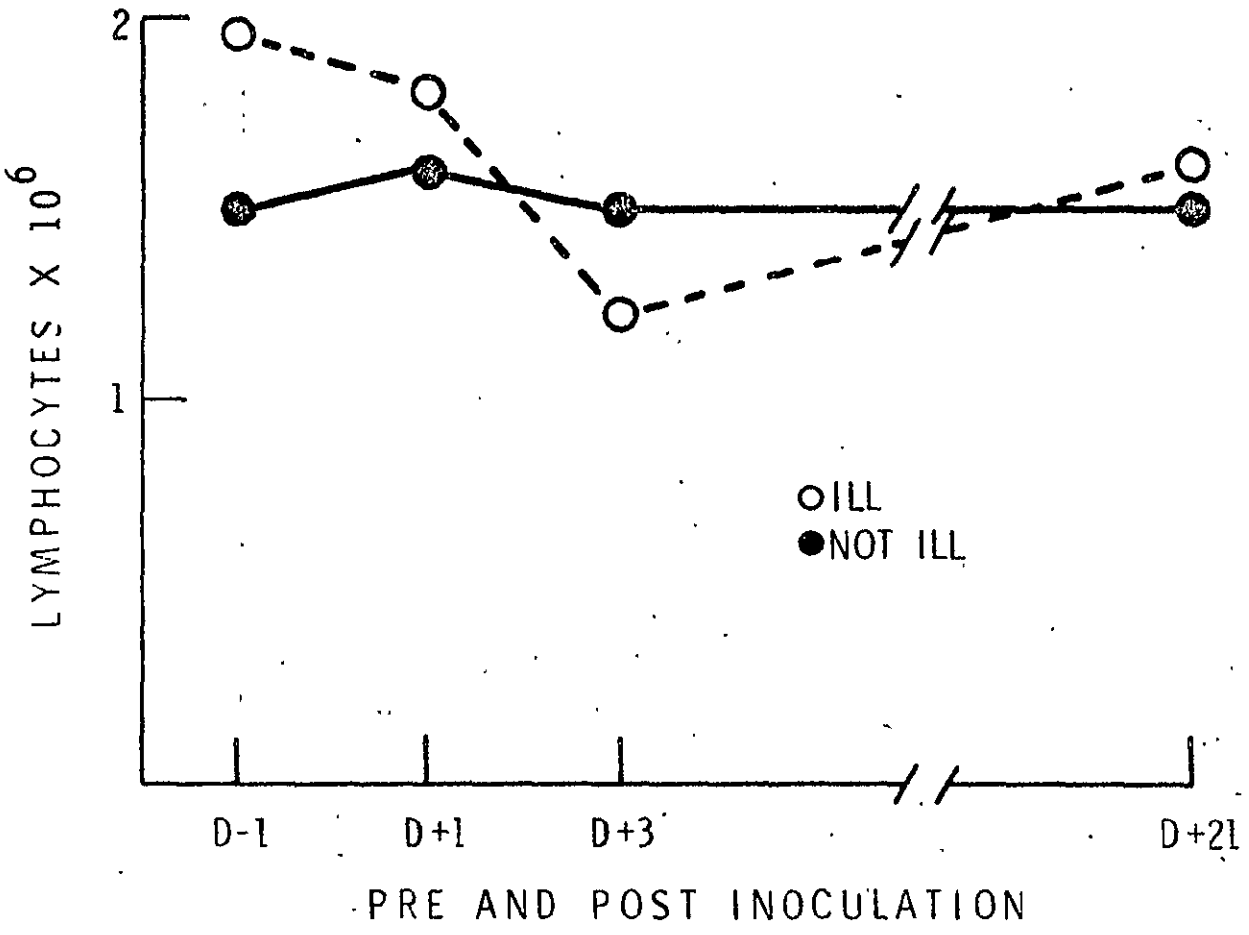
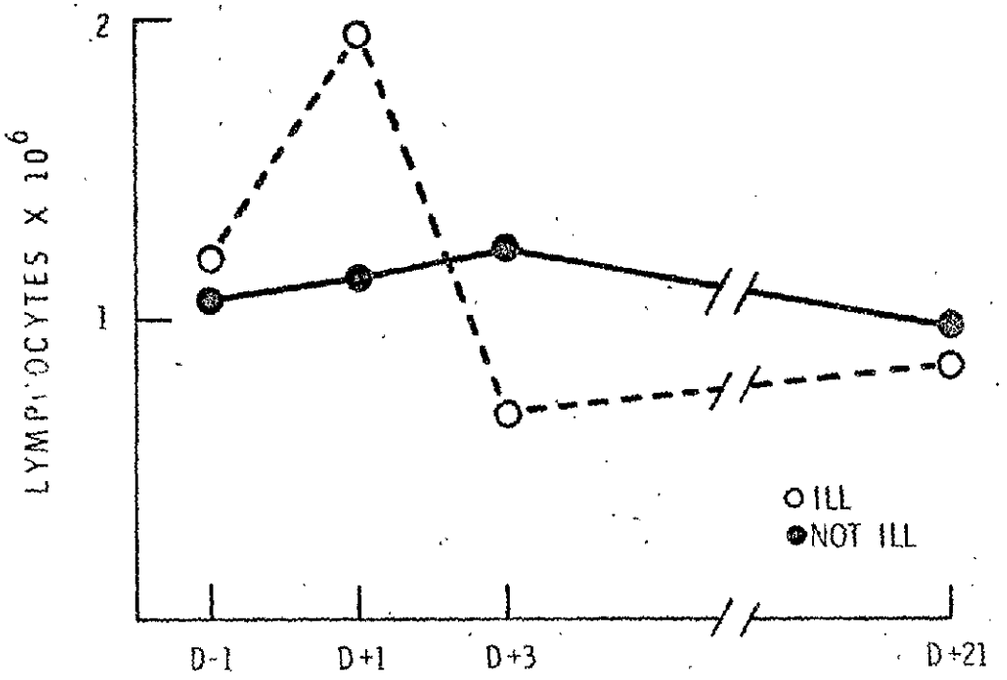


FIGURE 4

NON-REACTIVE LYMPHOCYTE RESPONSE
TO INFLUENZA ILLNESS



decrease ($P < 0.05$) to below normal on D+3 (0.68×10^6 to 1.04×10^6). The values at D+21 were significantly lower ($P < 0.05$) than the D-1 (0.86 to 1.21×10^6) although compared to normal values this difference was not significant. The initial rise seen at D+1 parallels that observed in the B cell population and the decrease seen at D+3 is consistant with the hypothesis that the non-reactive cells are undifferentiated B lymphocytes and may have, in response to this viral disease, completed differentiation.

We can summarize these findings as follows: total lymphocytes increase in the peripheral blood one day after challenge with influenza-virus, prior to clinical symptoms appearing. This increase was due to the increase in the B lymphocytes and the non-reactive cells. The total lymphocytes in the peripheral blood decrease three days following exposure to influenza virus, the time that symptoms begin to appear. This decrease is accounted for by a T cell and a non-reactive lymphocyte decrease.

In conclusion, changes in the lymphocytic population occurred prior to and concomitant with the appearance of clinical symptoms during influenza respiratory illness, suggesting that the study of these cells may be useful in detecting early viral infection.

III. A. Lymphocytes (continued).

3. Summary of Peripheral Blood Lymphocytic Response to Rhinovirus Infection and Mycoplasma Infections:

Subjects infected with Rhinovirus:

A preliminary study has been completed in which 5 volunteers were inoculated intranasally with Rhinovirus. Of these subjects 2 had no clinical illness and 3 were afebrile. All 5 had recoverable isolates. One subject who was in the ill group had an increase in white cell count and total lymphocyte numbers on day 2 following challenge. This lymphocyte increase was due to a T cell increase not a B cell increase. The other individuals showed no response or change from baseline. This same individual showed a distribution change on day 1 of an increase in IgM cells and a drop of IgG cells. IgA cells remained relatively stable.

Subjects infected with Mycoplasma pneumonia.

Twenty human volunteers were inoculated with either 1:50 or 1:500 dosage of Mycoplasma pneumonia PI 1428 by nasal drops and atomizer. The two dosages were identical in their effect upon the lymphocytic response. Two subjects dropped from the study before completion. No consistent trend appeared in the subgroup of B cells in contrast to the influenza study. An interesting generalization was noted, however, in comparing the numbers of lymphocytes/ml and the number of B cells/ml with the illness data.

Those subjects who became afebrile and/or ill (6 of the 20) had lower B cell and total lymphocyte counts than those subjects who showed afebrile conditions or no illness patterns. However, with this small of a sampling number, good statistical values showing significant changes were not available. In future studies this correlation will be further investigated.

A. Lymphocytes (continued).

4. Peripheral Blood Lymphocyte Response and Paraproteinemia during Severe Combined Immune Deficiency Disease: A State of Negative Functional Reactivity:

SUMMARY

A qualitative study was made of the plasma immunoglobulins of a child with severe combined immunodeficiency. By immunoelectrophoresis an immunoglobulin with an abnormal electrophoretic mobility was detected. This protein possessed μ heavy chain determinants, gave no detectable reaction with antisera specific for light chains, was of a relatively small molecular size, and was probably not composed of subunits held together by easily reduced disulfide bonds. The light chains that were present in this patient's plasma had a homogeneous electrophoretic mobility. The patient's plasma also contained at least two other immunoglobulins whose antigenic identity could not be established. One of these was abnormal in its electrophoretic mobility. The presence of the abnormal protein with μ determinants in the plasma of the second unrelated child with a similar disease suggests that the detection of this protein may have implications for the diagnosis or classification of immunodeficiency diseases.

INTRODUCTION

A frequent finding in immune deficient states is a quantitative change in the concentration of the serum immunoglobulins. Along with quantitative changes, the actual structure of the immunoglobulin may be changed yielding a paraprotein. Several descriptions of such proteins now exist (Radl et al., 1967; Schaller et al., 1966; Becroft and Douglas, 1968; Stoop et al., 1962). Paraproteinemias have reportedly been associated with such diseases as chronic lymphocytic leukemia in which immune function is also impaired. Two such cases described a μ (γM) heavy chain disease in which free heavy chains devoid of light chains appeared in a patient's plasma (Forte et al., 1970; Zucker-Franklin and Franklin, 1971). In both of these studies the levels of IgM paraprotein were increased above normal IgM values. In addition, a case of combined immune deficiency has been reported in which the patient possessed small numbers of lymphocytes bearing surface determinants specific for IgM heavy chain, but no light chains were detected (De Koning et al., 1969).

In the present study, the plasma immunoglobulins from a subject with severe combined immune deficiency were characterized quantitatively and qualitatively. The quantitative levels of immunoglobulins were

severely decreased in this subject, with IgM being the only one detectable at two years of age. IgA was never detected in the subject, and IgG declined to 0 over the two year period. The IgM that was detected was a paraprotein reactive only with μ antisera.

CASE STUDY:

A male infant that was suspected of having a severe combined immune deficiency disease was delivered by Caesarean section and maintained under gnotobiotic conditions as described previously by South et al. (1971). Prior to delivery of this child the subject's mother had lost a male infant at 6 months of age with severe combined immunodeficiency. The diagnosis of the subject studied in the present paper was confirmed by quantitation of immunoglobulins, challenge of his immune system in vivo with standard and experimental antigens and skin grafting, and in vitro testing of his lymphoid cells for reactivity to phytohemagglutin and pokeweed mitogen. X-ray studies provided no evidence for the presence of a thymus. The child had very low levels of circulating IgG and IgM, and no IgA detectable by quantitative precipitation techniques, and a diminished number of lymphocytes. During the course of a year and following multiple treatments with transfer factor, the patient developed two distinguishable types of lymphocytes, neither of which was totally normal structurally as seen by electron microscopy (Criswell et al., 1974, accompanying manuscript).

METHODS AND MATERIALS

Sample collection

Heparinized blood samples were drawn from the patient at intervals from age 9 to 22 months. Plasma was recovered by centrifugation and stored at -70°C until used. Plasma samples were also obtained from another immune deficient patient at 9 weeks and 18 weeks of age. This subject was a male who suffered from a similar combined immunodeficiency, was not gnotobiotic, and died of infection at 7 1/2 months of age.

Quantitation of immunoglobulins

To determine the quantity of IgA, IgG, and IgM present in the plasma, samples were tested (1) by radial immunodiffusion agar plates from Behring designed to detect quantities as low as 2 mg% and (2) by an electroimmunodiffusion method described by Gill et al. (1971).

Immunoelectrophoresis and chromatography

Immunoelectrophoresis was performed by the micromethod of Scheidegger (1955). Samples were subjected to electrophoresis in 1% agarose in barbital buffer (pH 8.2, ionic strength 0.025) at 35 mA, 10 volts/cm for 45 minutes. Sample volumes used were

2 μl of normal plasma and 6 μl of the patient's plasma that had been concentrated approximately 10 times at 4°C with Lyphogel (Gelman Instrument Co.). Precipitin bands were developed for 24 hours with 0.1 ml of one of several commercial antisera to human plasma proteins (Behringwerke). In addition, some experiments used unpooled rabbit antisera prepared against the patient's own plasma emulsified with an equal volume of Freund's complete adjuvant.

Gel filtration chromatography of normal plasma and the patient's plasma was performed using Sephadex G-200 (Pharmacia) in phosphate-buffered saline, pH 7.2, in a column with inner diameter 1.6 cm, and length 89.2 cm.

Fluorescent immunoglobulin staining for B lymphocytes

Leukocytes were obtained as a buffy coat preparation from heparinized whole blood following centrifugation at 600 g for 15 min. The RBC's were removed by agglutination with appropriate typing sera. No lymphocytotoxic activity was present in these pretested antisera. Following agglutination the sample was divided into two parts. Part A was directly stained with fluorescein conjugated antihuman polyvalent antisera (Meloy Labs) to determine the number of B lymphocytes present. After staining

for 30 minutes at 4°C, the preparation was washed three times with cold phosphate buffer saline (PBS) at pH 7.4. Slides of the preparation were examined on a Leitz Ortholux photomicroscope equipped with an HBO 200 mercury burner and a ploem illumination system.

RESULTS

Table 1 gives the quantitative values of the subject's plasma immunoglobulins from the age of 2-25 months. IgA was never demonstrated by either method employed. IgM was present from 2 months to 11 months at detectable, though very low levels. At 17 months a slight increase was noted, yet IgM was still quantitatively very low. This level of IgM was maintained through 25 months. IgG concentrations were within normal ranges at birth and gradually fell to 0 at 20 months of age, indicating that probably all the IgG was transplacentally transmitted and presumably maternal IgG.

Immunoelectrophoresis of the patient's plasma with a polyvalent antiserum to human IgG, IgM, and IgA revealed at least four precipitin bands (Fig. 1A & 1C). Two of these bands had electrophoretic mobilities largely within the normal range for immunoglobulins, but the two other bands showed an abnormally fast migration. When

TABLE 1. Plasma immunoglobulin levels in mg percent

Age of Subject	IgG	IgA	IgM
2 months	235	0	7.0
3 months	174	0	5.6
4 months	130	0	5.0
6 months	82	0	8.8
7 months	53	0	5.0
8 months	50	0	3.6
11 months	17	0	6.9
17 months	N.D.	0	13.7
18 months	7	0	15.9
20 months	0	0	14.4
21 months	0	0	13.7
25 months	0	0	15.2

N.D. = Not Done

monospecific antisera to either IgG, IgM, IgA, or K or λ light chains were used, only one reaction could be detected: antiserum to IgM precipitated one of the abnormally-migrating bands, indicating the protein represented by this band possessed μ heavy chain determinants. No IgM of normal electrophoretic mobility was demonstrated in the patient's plasma. All samples from this patient, including one obtained prior to his treatment with transfer factor, contained the abnormal μ determinant protein. A similar band was found when samples of plasma from the second immunodeficient child were examined.

Analyses were next done to determine if the abnormal μ determinant protein might represent an artifact of preparation. When normal plasma was subjected to the same concentration procedure with Lyphogel, no abnormal protein could be detected, indicating that the paraprotein was not a procedural artifact. Furthermore, one volume of the patient's plasma was added to 4 volumes of normal plasma, and the mixture was incubated at 37°C for 45 minutes to determine if the paraprotein could represent a degradation product. Immunoelectrophoretic analysis after incubation revealed no degradation of the normal plasma immunoglobulins indicating further that this protein was not a degradation product of a species of normal immunoglobulin caused by an enzyme in the patient's plasma.

The failure of the remaining proteins detected with polyvalent antiserum to precipitate with the various monospecific antisera may represent further abnormality of this child's immunoglobulins.

Of the two proteins in the patient's plasma with a more normal electrophoretic mobility, one was seen to produce a precipitin arc with a distorted shape. Since the polyvalent antiserum used to precipitate these proteins also precipitated from plasma a band in a similar position which was neither IgG, IgM, nor IgA it is possible that one of these two bands represents a 7s IgM that Soloman (1969) has reported in normal sera.

Neither free K or λ light chains could be detected by immunoelectrophoresis even when a 30-fold concentration (6 μl sample volume) of the patient's plasma was examined. A small bowed distortion near the antigen origin appeared in both the normal K or λ arcs (Fig. 2) when the patient's concentrated plasma was mixed with normal plasma prior to immunoelectrophoresis. Thus, the patient's plasma does contain some molecules bearing K and λ determinants but having a very restricted electrophoretic mobility.

Table 2 summarizes the results found upon examination of the patient's lymphoid cells for surface immunoglobulins containing light chains. The cells possessed both heavy chain and light chain determinants. As can be seen, approximately 60% of this subject's lymphoid cells possessed surface immunoglobulins, that is, they are B lymphocytes.

TABLE 2. Immunoglobulin specificity of B lymphocytes

Fluorescent Antibody Stain Specificity	Percent Cells Staining
IgG (γ specificity)	22%
IgM (μ specificity)	32%
IgA (α specificity)	7%
total	61%
IgG + IgM + IgA (polyvalent antiserum)	60%
K and λ type light chains Δ	64%

Δ indirect fluorescent antibody technique

The patient's plasma proteins contained no antigenic determinants that were unique only to himself, as indicated by immunoelectrophoresis of the subject's plasma with the rabbit antisera produced against the patient's own plasma. When these antisera were absorbed with normal plasma, no precipitation of the patient's plasma was detected.

To estimate the molecular size of the subject's immunoglobulins, whole plasma and a protein fraction precipitated by one-third saturation with ammonium sulfate were subjected to gel filtration chromatography with Sephadex G-200. Two elution peaks, having elution volumes identical to the "4S" and "19S" peaks of normal plasma, were obtained using the patient's plasma. The "7S" peak was absent. The abnormal μ determinant-bearing protein was recovered from the ammonium sulfate precipitated material in the "4S" peak, indicating that this protein is much smaller than normal IgM. No IgM activity was detected in the "19S" peak. Immunoelectrophoresis of the eluted fractions did not reveal any other immunoglobulins.

Normal serum was treated with 0.1 M 2-mercaptoethanol prior to immunoelectrophoresis and changes in the immunoelectrophoretic band position of subunits of IgM were noted. The arc representing IgM was precipitated very close to the antibody trough because of the enhanced diffusion of the smaller IgM subunits. Such treatment of the patient's plasma produced no displacement of the arc with μ specificity, indicating that this paraprotein is probably not formed of subunits aggregated in the manner of normal IgM.

DISCUSSION

The plasma of this child with severe combined immunodeficiency contains at least one qualitative immunoglobulin abnormality. Its small molecular size and lack of light chain reactivity suggest that the molecule which reacts with antiserum to IgM is a μ heavy chain or heavy chain fragment. An additional abnormality is suggested by the fact that the patient's light chains, which could be detected only indirectly, appeared to be very homogeneous in their electrophoretic mobility.

The presence of an abnormal circulating immunoglobulin suggests a number of questions. The molecules of IgM of this patient may reflect faulty protein synthesis, or they may undergo degradation during secretion from the plasma cell. Through our determination, it was impossible to determine whether the light chains, which we can demonstrate by fluorescent antibody staining of the surfaces of the lymphocytes, are present in normal quantities and are normally bound to heavy chains.

The first child of this study has been maintained in a germ-free environment since birth and has never been ill. The second child was not so protected, became infected, and died. Both had the μ paraprotein, suggesting that the presence of this abnormality is not

dependent on the clinical status of the patient and is not a product either of bacterial degradation or of alteration of the immune system due to infection. Both their immune systems at birth appeared very rudimentary and undeveloped.

Since this disease may represent a unique stage of development, one might question whether the release and the slight observed increase of this IgM fragment represents the very beginning of a phase of development in the immune system, or whether this child's immune system is nonfunctional as a result of a severely inhibited cellular synthetic mechanism. The lymphocytes have both the light (L) chain and heavy (μ) chain determinants present; yet the plasma contains μ chain fragments and electrophoretically abnormal L chains. Buxbaum and Scharff (1973) have described in detail the probably synthetic events that lead to IgM production. The μ chains have been shown to be synthesized on ribosomes, released into the cytoplasm free, and then connected later by disulfide bonds to L chains. Additionally, Schubert (1970) has shown that in the course of a short-term pulse-labeling experiment, a polypeptide can be identified that precipitates with anti-μ antiserum, is small in size, and lacks carbohydrate. Buxbaum and Scharff (1973) have shown that this smaller μ fragment is the precursor of the larger intact μ chain. This smaller polypeptide might be the same as the

abnormal μ determinant protein found in the present study. The μ fragment might be leaking to the outside of the defective lymphoid cells of this subject.

In addition, the fact that the paraprotein found in this child is of a μ specificity is consistent with the possibility that his immune system may represent a very early developmental stage of the immune response of man. IgM appears to be the first immunoglobulin produced during ontogenesis of man (Adinolfi & Wood, 1969), as well as other mammals (Chapman et al., 1974) and the chicken (Kincade & Cooper, 1971).

A second unrelated child also was found to have the 4S μ paraprotein. Both subjects had the sex-linked form of severe combined immune deficiency. The presence of the paraprotein in this second child suggests that the detection of this protein may be of diagnostic value and might represent a serologic marker for this disease.

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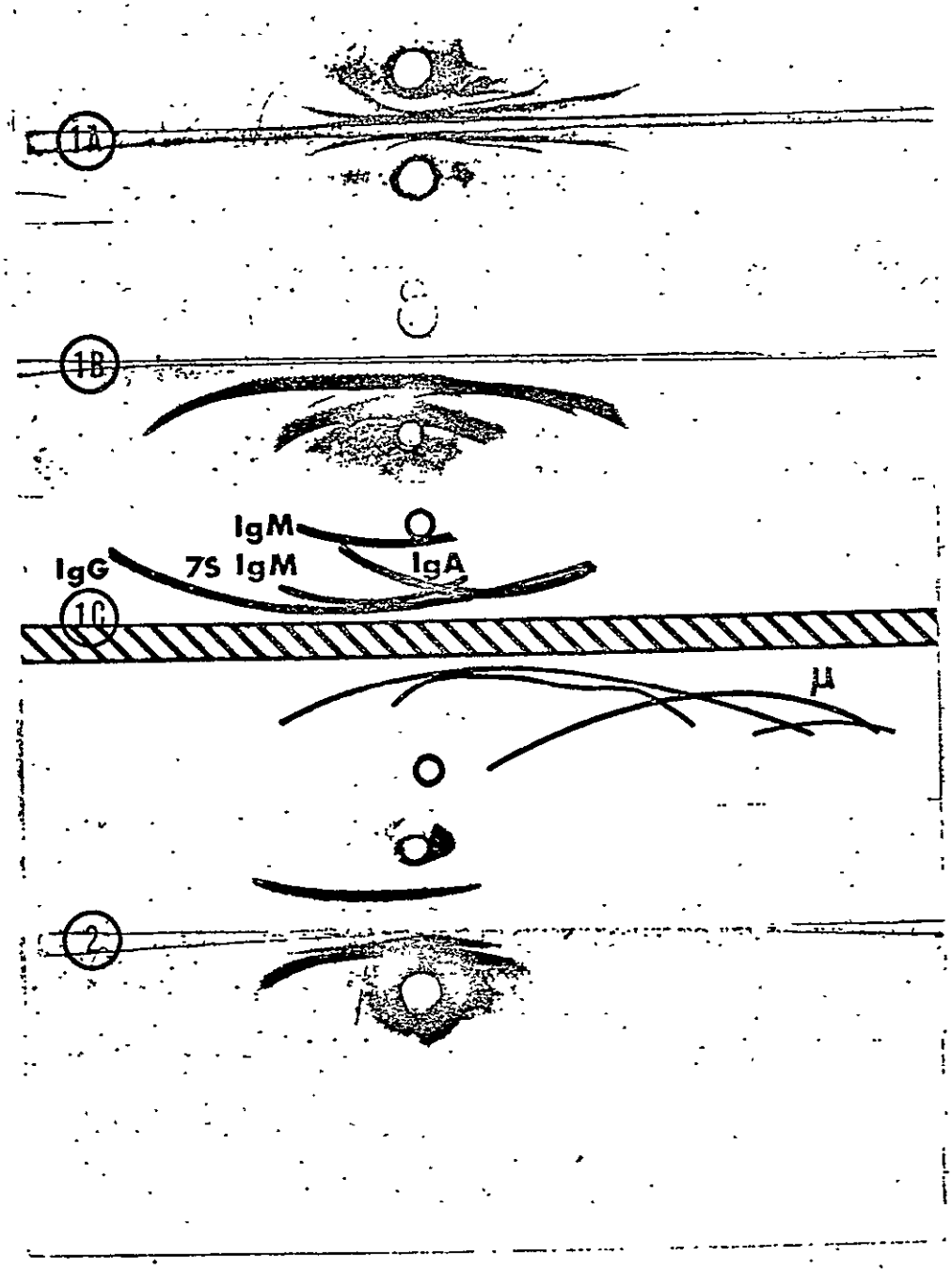
FIG. 1. a. Immunoelectrophoresis of concentrated plasma from a child with severe combined immunodeficiency (upper and lower wells) with antiserum to human IgG + IgM + IgA (trough). b. Control consisting of normal adult plasma (lower well) and antiserum to IgG + IgM + IgA (trough), included in the same electrophoretic run as a. c. A diagrammatic representation of the reactions shown in 1a (lower well) and b (upper well). The band marked "μ" represents the protein which is precipitated by antiserum to IgM.

FIG. 2. Immunoelectrophoresis of normal adult plasma (upper well) and normal plasma plus plasma from the immunodeficient patient (lower well) with antiserum to light chains (trough). Note the distortion of the lower arc near the antigen origin.


1A
1B
IgM
IgG
7S IgM
IgA
1C
μ
2

A. Lymphocytes (continued)

5. Microspectrophotometry of Peripheral Blood Lymphocytes:

Present Goals:

The following study was conducted for these purposes: (1) to determine what is a "normal" peripheral blood lymphocyte, in terms of detailed morphological features not discernable by the naked eye. This was accomplished by observing the internal DNA-RNA-protein complex structure of individual lymphocytes by the technique of scanning microscope photometry. Based upon this information, "normal" lymphocytes were induced into blastoid transformation to "observe" the immunological reaction, i.e., the dynamic morphological changes taking place inside each cell. At the same time, an estimate was made of the percent of lymphocytes which did undergo blastoid transformation as a result of pokeweed mitogen stimulation. This data was correlated with concomitant DNA and RNA synthesis studies and assumed protein synthesis to gain further insight into morphological biochemical relationships.

(2) "Normal" lymphocyte optical image patterns were then compared to lymphocyte patterns from two individuals known to have severe combined immunodeficiency disease (SCID). In one SCID case, a distinct trend toward "normal" was observed after the administration of transfer factor, although all other means of testing proved negative.

This not only suggests the possibility that this technique may be highly sensitive to minute morphological, and possibly biochemical, changes, but also shows promise as an aid in monitoring the difficult treatment of these individuals. This was suggested by the other case of SCID which presented a completely different picture, indicating that severe combined immunodeficiency disease is not just one entity but possibly many shades of gray. A precise assessment of the disease could very decidedly influence the course and type of treatment tailored to the individual.

Completed Study:

Introduction

The immune system of man, composed of lymphoid organs, tissues and lymphocytes, is designed to protect an individual against disease by either producing antibodies which neutralize or destroy the challenge or by eliciting a direct cell-to-cell cytopathic effect upon the challenge. According to Miller and Osoba (1967) the capacity to effect an immune reaction coincides with the appearance of lymphocytes circulating in the peripheral blood.

Since the early 1950's much knowledge of the immune system has been gained through a few key observations. The first recorded case of agammaglobulinemia by Bruton (1952) gave researchers their first clue to the correlation of immune system disorders and clinical illness.

In 1953 Good conducted experiments to determine if the thymus produced antibodies but found no effect of thymectomy on adult or young rabbits. In 1956, Glick reported that the removal of an organ called the bursa of Fabricius in young chickens markedly affected antibody production. This was supported by studies by Chang et al. (1957, 1958, 1959) which showed considerable impairment in the capacity to produce antibody after surgical removal of the bursa of Fabricius but inconsistent effects on antibody production after neonatal thymectomy (Good, 1962, Hess and Stone, 1963, Basch, 1966 and Miller and Osoba, 1967).

In 1955 Cooper described the presence of two distinct organs in the chicken, the thymus and the bursa of Fabricius, and suggested differences in function for these two organs. He and other investigators showed that cell-mediated immune responses, such as delayed hypersensitivity and transplant organ rejection, were impaired after thymectomy (Good et al., 1962 and Martinez et al., 1962). Warner and Szenberg (1964) showed that antibody-producing lymphocytes came from the bursa of Fabricius and, consequently, that bursectomized chickens lacked serum IgG (Carey and Warner, 1964), and if sublethally irradiated, also lacked serum IgM (Cooper et al., 1965). Cooper (1966a and b) later showed that a clear-cut separation of immunologic function did

indeed exist in the chicken. The thymus appeared to be the site of differentiation of small lymphocytes involved in cell-mediated hypersensitivity reactions while the bursa was the site of antibody-producing large lymphocytes.

Miller and Osoba (1967) theorized that the critical period when the thymus, and possibly the bursal equivalents, suggested to be the appendex, Peyer's patches, and/or the bone marrow, influenced immunological competence of lymphocytes occurred during early life while the lymphoid tissues were developing and immunologic capacity was maturing.

Assimilating this data, Good (1971) hypothesized that the immune system consisted of stem cells in the bone marrow migrating to certain primary organs, the thymus and bursa or bursal equivalent(s), to produce lymphocytes, which then seeded to secondary organs and tissues. This hypothesis was based on studies of the immune system by many investigators (Gesner and Gowans, 1962, Micklem et al., 1966 and McGregor, 1968). The thymus has been shown by Miller and Osoba (1967) to constantly produce large numbers of small, immunologically mature lymphocytes involved in cell-mediated responses; disturbances of the thymus have been known to be associated with immunological defects. The first unequivocal evidence for an immunologic function of the small lymphocyte came from studies on the

immunology of tissue transplantation (Billingham et al., 1959). It has been stated that the functional immunologically-competent component of the immune system is the lymphocyte (Craddock, et al., 1971a and b).

There are two main classifications of lymphocytes: T and B lymphocytes. The T lymphocytes are those small cells which have been "differentiated" by the thymus gland, hence the term thymus-derived or T cells. The B lymphocytes are larger cells released from the bone marrow and seeded to lymphoid organs and tissues without thymic differentiation, hence, the term B cells.

T lymphocytes have been shown to differentiate either within the thymus itself or outside the thymus gland possibly under the influence of thymosin, a thymic hormonal factor, and other hormones according to Goldstein (1971). Owen and Raff (1970) have shown that this differentiation and maturation process occurs in two stages: the first being aquisition of a specific surface alloantigen, theta, with the subsequent appearance of histocompatible antigen, HL-A or H-2. In the rat, thymic cortical lymphocytes have been described as dense, cortisone-sensitive, theta positive, HL-A or H-2 negative and immunologically incompetent, whereas thymic medullary lymphocytes have been described as more buoyant, cortisone-resistant, theta negative, HL-A or H-2 positive and immunologically active (Raff and Cantor, 1971).

In the peripheral blood, a small percentage of T lymphocytes have a short life span ranging from hours to days while the majority are small, recirculating cells of long life span of from months up to 10 years (Everett, 1964 and Buckton and Pike, 1964). T lymphocytes are believed to contain antigenic receptors on their cell surfaces. Recent studies by Feldman et al. (1973) show that monomeric IgM, both light and heavy chains, is present on activated T lymphocytes. They suggest that the T lymphocytes with monometric IgM serve two roles: (1) reception of antigen and (2) transmission of immunologic information allowing T lymphocyte cooperation with B lymphocytes.

The T lymphocytes are believed to confer cell-mediated immunity as specified by delayed hypersensitivity, homograft rejection and graft versus host reactions (Gowans, 1962 and Good, 1971). Immune surveillance by T lymphocytes against neoplasms has also been suggested (Miller and Osoba, 1967, Craddock, 1971 and Klein, 1973). These cells have also been shown to contain "memory" (Gowans, 1971). After a T cell has produced its primary response against a particular antigen, any subsequent exposure to the same antigen would elicit a secondary response requiring less time to produce a stronger reaction. These cells secrete soluble substances such as mitogenic, chemotactic and cytotoxic factors, in addition to

migration inhibitory factor (MIF), transfer factor and interferon (David, 1971). These factors are collectively known as lymphokines. Dumond (1970) has analyzed some of these factors in man and found them not to be immunoglobulins.

B lymphocytes in the peripheral blood are believed to be of medium-to-large size with a short life span (Craddock, 1965). Many investigators believe that the cell which emerges from the bone marrow is the functional B lymphocyte while others suggest that the B lymphocyte must mature after leaving the marrow. The maturation initiator is unknow but a bursal hormone has been postulated.

Many B lymphocytes contain immunoglobulins on their cell membranes, the presence of which is detectable by immunofluorescent and other staining techniques (Raff, 1970). Rabellino et al. (1970) found in mice that between 50,000-150,000 molecules of immunoglobulin are present on each positively staining B lymphocyte. Some investigator believe actively synthesizing B cells transform into plasma cells (Gowans & Knight, 1964). Other B lymphocytes synthesize and secrete large amounts of immunoglobulin molecules into the blood. These immunoglobulins are of five classes: IgG, A, M, D and E. Their role is to attach to specific antigens, hence they are termed serum antibodies.

The B lymphocytes are responsible for the humoral phase of immunity Serum antibodies have been shown

against infection. Serum antibodies of the IgG and IgM classes have been shown to participate actively against viral and Gram-negative bacterial antigenic challenges while antibodies of the IgA class have been shown to line and actively participate against infections along the mucous membranes of the body.

Stated very simply, the lymphocytes are the "responders" of the immune system. Studies by Ada (1967) show that when an antigen enters the body, it is carried via lymphatics to regional lymph nodes where it evokes a local reaction, the principal finding of which is cellular inflammation (Waksman, 1960). Long-lived recirculating lymphocytes in the cortical region of the lymph node then migrate out and populate distal lymph nodes to promote a systemic reaction. These cells may be observed in the peripheral blood as they circulate to distal lymph nodes and tissues (Burnet, 1970). Lymphocytic response results in two types of immunity: humoral and cell-mediated. Upon antigenic stimulation, both the T and B lymphocytes will respond, but one type may be favored over the other; or they may act in concert to produce an optimum response, depending upon the antigen. There is evidence that some antigens, such as heterologous erythrocytes, require B and T lymphocyte interaction to produce optimum immune response (Davies, 1967, and Miller, 1971).

Schechter et al. (1973) and Levy and Rosenberg (1973) have shown that activation of lymphocytes by phytomitogens, termed blastogenesis or blastoid transformation, causes changes in membrane transport, enzyme levels, gene activation and non-specific protein synthesis. In addition, blastogenic changes are accompanied by changes in cell size and DNA and RNA synthesis (Chessin et al., 1966, Douglas et al., 1966 and 1969 and Bartels et al., 1969).

By the use of scanning microscope photometry and radioisotope incorporation studies, those blastogenic changes resulting from lymphocyte activation as reported by other investigators were confirmed and additionally clarified in greater detail on cell by cell level.

METHODS AND MATERIALS

Specimen Analysis

Peripheral blood lymphocytes from a normal adult and a normal baby 20 months of age, selected on the basis of good health and no apparent illness, and two babies diagnosed as severe combined immunodeficiency were analyzed in detail by the technique of scanning microscope photometry (SMP).

Lymphocytes from three additional subjects were analyzed by scanning microscope photometry (SMP) for the establishment of a "normal" blastogenic response curve following Pokeweed mitogen (PWM)

stimulation and analyzed for DNA and RNA synthesis rates using a standardized radioisotope incorporation procedure.

Blood Collection

In order to perform DNA and RNA synthesis studies on lymphocyte cultures, whole blood was obtained by venipuncture and placed in sterile 100 x 16 mm Vacutainer tubes containing sodium heparin (0.2 ml to 10.0 ml whole blood, 200 units) to prevent clotting. Generally, a yield of 1×10^6 lymphocytes/ml of whole blood was obtained.

Plasma Immunoglobulins

Plasma immunoglobulin levels were performed to determine the severity of the severe combined immunodeficiency subjects. Plasma IgG, IgA and IgM levels were obtained for the normal subjects using the procedures recommended by Meloy Laboratories. Plasma immunoglobulin levels from the immunodeficient babies were determined by the method of Gill et al. (1971) with slight modifications including running the IgG procedure for 75 minutes instead of 60 minutes and performing the IgA test using the barbital system instead of the phosphate system as described by Gill.

Lymphocyte Separation

Lymphocyte separation was accomplished by the Technicon automatic lymphocyte separator method (Technicon, 1971) as previously described.

At this time a lymphocyte wet mount preparation was observed under the microscope at 45X to determine the purity of separation and also to check for any gross cell abnormalities. The purity always ranged above 97% lymphocytes and no gross morphological abnormalities were found.

Lymphocytes to be used for culturing were washed 3 times in sterile MEM-Spinner modified without L-glutamine (Gibco #138) in sterilized Fisher tubes and centrifuged at 2,500 g for 2 minutes in a Fisher Centrifuge to obtain a pellet. Lymphocytes with no culture requirement were transferred to Fisher tubes, washed 3 times in cold phosphate buffered saline (PBS) and centrifuged at 2,500 g for 2 minutes.

B Lymphocyte Determinations

The percentage of separated lymphocytes containing membrane-bound immunoglobulins was determined for possible correlation with mitogenic response. Approximately 1×10^6 lymphocytes were stained with 3 drops of a 1:2 dilution of either fluorescein-conjugated anti-human IgG, anti-human IgA, or anti-human IgM antisera (Meloy Laboratories, prepared in goats, 10 mg/ml. fluorescein-isothiocyanate/protein (F/P) ratio = 2.2 µg/mg) at $4^{\circ}C$ for 30-60 minutes. The cells were then washed 3 times with PBS to eliminate any non-specific stain and the supernatant

removed. One drop of the cell suspension was placed on a microscope slide, a cover slip added and sealed with nail polish. The B cell percentage was determined by counting the number of fluorescent cells per 100 lymphocytes under visible and fluorescent light on a Leitz Ortholux microscope equipped with an HBO 200 Mercury burner and a ploem illuminator with a S 360 (UG 1) UV exciting filter, a K 460 barrier filter, and a S 525 interference filter (fluorescence selection filter).

Culture Technique

Lymphocyte cultures were set up by the method of Curtis et al. (1970) as previously detailed in the Feb.-April, 1974 Progress Report. Lymphocytes were stimulated with 0.05 ml Pokeweed mitogen (PWM) containing 250 µg (Gibco #536) to give a final concentration of 82 µg PWM/ml tissue culture media. The 16 x 125 mm sterile culture tubes were incubated at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

Pokeweed mitogen was chosen as the non-specific mitogen for two reasons: (1) it produces less clumping than phytohemagglutinin (PHA), enabling better slide preparation for SMP scanning, and (2) although not as potent a mitogen as PHA which induces 70% or more lymphocytes to undergo blastogenesis, PWM affects a wider spectrum of lymphocytes, stimulating both mature T and B lymphocytes.

Tritiated Thymidine and Uridine Incorporation Measurements

DNA and RNA incorporation studies were performed by the method of Hersh (1971) to aid in interpretation of blastogenic changes as defined by scanning microscope photometry. This procedure also was previously described in detail in the Feb.-April, 1974 Progress Report.

Nucleic Acid/Protein Analyses

At 3, 5 and 7 days following the addition of pokeweed mitogen, cultured lymphocytes were harvested and washed 3 times in PBS. A drop of the cell suspension was placed on a Quartz glass slide and allowed to air dry. The slide was then fixed in absolute ethanol for 15 minutes, rinsed with tap water, set upright and again allowed to air dry. The slide was then cover-slipped, covered with a Quartz cover-slip using immersion glycerin (refractive index 1.459, 20°C, Carl Zeiss) as the mounting medium and sealed with clear nail polish. The slide preparation was then ready for scanning microscope photometry (SMP) analyses. The scanning microscope photometer (SMP) was connected on-line to a Digital PDP-12 computer to obtain nucleic acid/protein measurements within individual lymphocytes. The system consisted of the following major components: (1) a Zeiss universal microscope equipped with a high-speed mechanical stage and Ultrafluor objective lenses for UV scanning, (2) a Xenon 150 W lamp with M4QIII prism

monochromator and light modulator for achievement of monochromatic light at 280 nm, (3) an amplifier for analog to digital conversion housed in an electronic module with stabilized high voltage and adjustment control knobs for 0-100% T, (4) a Teletype for input of program instructions and (5) a Digital high-speed reader for output of data.

Figure 1 shows a simplified light beam schematic for the SMP system. Using the 100x Ultrafluar objective lens and 12.5 Kpl eyepiece, individual lymphocytes were positioned for scanning. The light beam was then centered and adjusted under visible light at approximately 540 nm. The beam was condensed to 2 μ and directed through the condenser under the high-speed mechanical stage. With the measuring spot set at 0.5 μ and the field stop aperature closed, the monochromator was turned to 280 nm, the light beam directed by the single beam splitter to the protomultiplier and the percent transmission slopes adjusted to 0% and 100% T. Scanning was initiated by giving commands via the Teletype to the Apamos II command program magnetic tape (Carl Zeiss).

When the command was given to initiate scanning, pulses of light were emitted and the absorbed light was directed to the beam splitter for PMT signal measurement. Pulsing stopped to allow the high-speed mechanical stage to move 0.5 μ. Absorbance measurements were taken

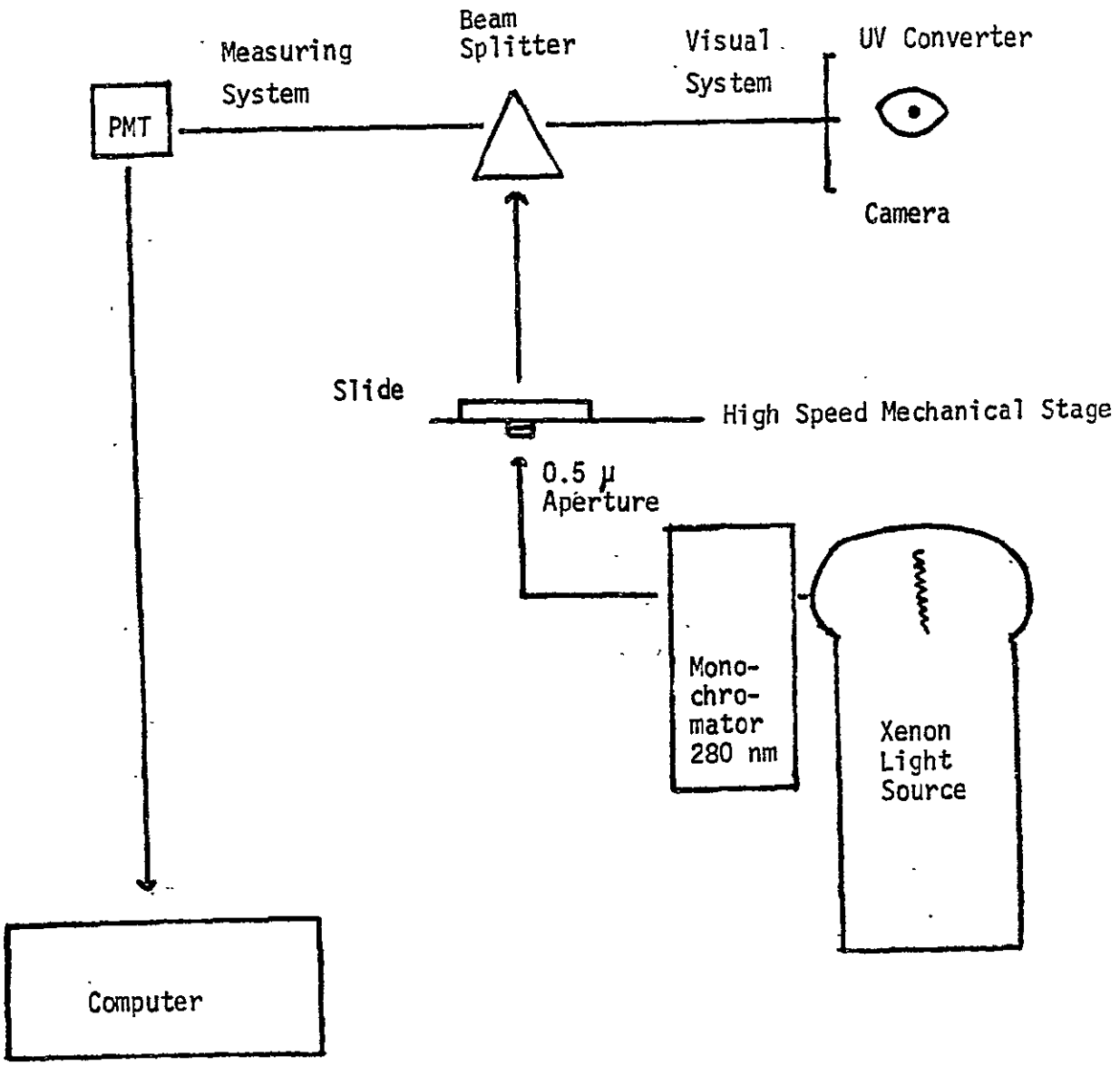


FIGURE 1. Simplified Light Beam Schematic for SMP System.

at 0.5 μ intervals across a cell until the specified area to be scanned was covered. The time required to scan a cell was approximately 3 minutes.

After an entire cell was scanned, the 0-100% T slopes were checked for drift and, if negligible, the absorbance data was kept and stored on a magnetic tape by the computer. Individual cells were given a code name, any debris surrounding the cell removed and the data filed on a blank tape. In this manner, approximately 55 cells per slide were scanned (Zeiss and Apamos II operator instruction manuals). An APCVRT program tape written in-house was used to convert data from the Apamos II tape into a form acceptable to TICAS program tapes (Wied and Bahr, 1970). TICAS programs were then used to view, clean, reduce data to meaningful parameters and print out both raw and reduced data on a Digital high speed reader.

Figure 2 shows an example of a raw printout for a normal lymphocyte. Each circle represents a partial optical density value, in absorbance units, at 0.5 μ intervals across a cell.

The total number of these circles determines the relative cell area which is related to cell area. The greater the number of circles, the larger the cell. The diameter of any cell (assuming it is round) can be calculated using the formula $A = \pi r^2$ or $d = 2 \times \frac{A}{\pi}$.

SCUN0056

Measured Circles = 196

$$d = 2 \times \sqrt{\frac{A}{\pi}}$$

$$A = \frac{196}{4}$$

```
                    6  9 12 12  8  3
                 7 14 18 24 27 24 19 12  8
              11 19 25 26 29 31 34 28 24 20 11
           10 23 27 33 34 31 32 36 32 30 26 19  5
           18 28 32 34 36 37 38 41 41 39 31 24 14
        8 21 28 33 36 40 43 43 44 45 38 33 27 19  8
       12 23 29 36 39 43 48 46 46 43 36 32 27 20 10
       12 21 33 40 41 43 47 46 40 36 35 33 28 20 10
       11 23 30 35 43 43 43 43 39 35 32 31 28 20  9
       14 25 28 31 35 38 41 41 39 36 31 30 26 17  4
        9 21 28 29 31 34 39 39 36 33 31 28 20 16  3
        4 15 26 28 31 34 36 36 33 32 28 25 17 11
           8 18 26 30 31 31 30 31 30 24 21 16  7
              7 15 22 23 24 26 27 27 19 18 11  3
                 5 10 12 16 19 19 17 12 12  3
                       5  8  9  7  3
```

FIGURE 2. Example of a Computer Raw Printout.

Table I compares relative cell area to both cell area and cell diameter.

r = radius

A = area (relative cell area)

$= 3.1416$

The summation of the partial optical density (absorbance) values results in the total optical density value. This gives an indication of the nucleic acid/protein content. It is expressed on the reduced data printout in absorbance units termed the total extinction, as 4.989 + 003. The mean frequency, or mean (average) O.D., is calculated by dividing the total extinction (absorbance) by the relative cell area (number of circles 0.5 μ in diameter).

Figure 3 shows an example of a computer reduced cell printout. The 5 parameters employed for analysis of lymphocytes included: area (relative cell area), total extinction, mean frequency, sum of 5 highest values frequency and sum of 5 highest values difference.

Table II compares the computer language to common terminology and describes each parameter employed. Area (relative cell area) is related to cell area and cell diameter as previously stated. Total density (extinction) of a cell is, according to Sandritter (1970), the staining intensity measured at an absorption maximum. Our measurement of unstained lymphocytes at 280 nm resulted in measurement of

TABLE I

Relationship of Relative Cell Area

to Cell Diameter and Area

Relative Cell Area (Circles)	Diameter (μ) $d = 2 \times \sqrt{\frac{A}{\pi}}$	Cell Area (μ^2) $\frac{\text{Circles}}{4}$
114	6	28.5
154	7	38.5
202	8	50.5
315	10	78.8
454	12	113.5
616	14	154.0

NAME SCUN 0056

AREA (RELATIVE CELL ARES	196
TOTAL DENSITY (ABSORBANCE)	+4.9890008 + 003
MEAN DENSITY (MEAN CELL DENSITY	26
MEDIAN FREQUENCY	27
MEAN DIFFERENCE	5
MEDIAN DIFFERENCE	5

SUM OF 5 HIGHEST VALUES FREQUENCY	233
SUM OF 10 HIGHEST VALUES FREQUENCY	431
SUM OF 5 HIGHEST VALUES DIFFERENCES	73
SUM OF 10 HIGHEST VALUES DIFFERENCES	136

FIGURE 3. Example of a Computer Reduced Printout

TABLE II

Interpretation of Computer Terms

Computer Language	Common Terminology	Description
Area	Relative cell area	Related to cell area and cell diameter
Total Extinction	Total density (in absorbance units)	Sum of all the partial optical densities (NA/P content)
Mean Frequency	Mean optical density or mean cell density	Average optical density or average cell density
Sum of 5 Highest Values Frequency	5 highest values	Sum of the 5 highest partial O.D.'s (related to NA/P composition)
Sum of 5 Highest Values Difference	5 highest values difference	Sum of 5 greatest differences between partial O.D. values (indicates NA/P heterogeneity or distribution

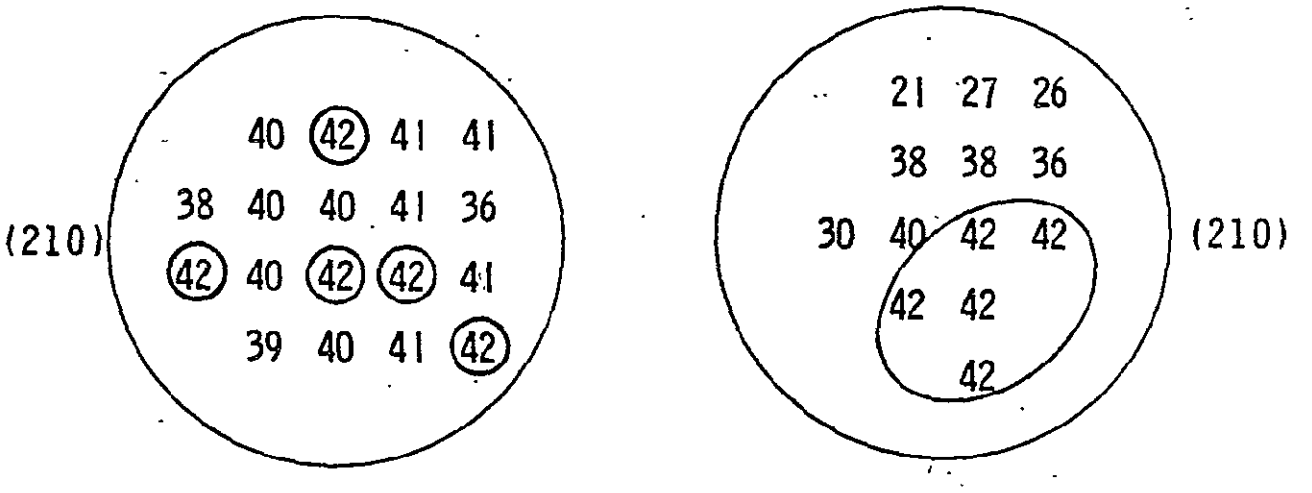
NA/P = Nucleic acid/protein.

both nucleic acid and protein since these components could not be separated due to the nature of the material. Similar studies by Wied et al. (1968) showed that no additional information was gained by scanning at 260 nm, the differences being quantitative rather than qualitative.

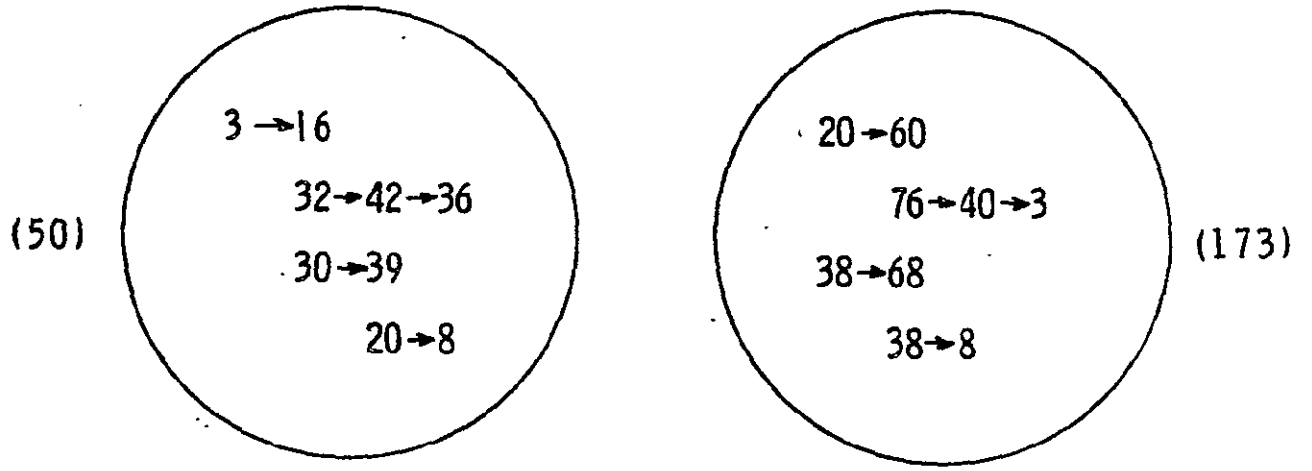
Figure 4 aids in the explanation of the Sum of the 5 Highest Values Frequency and Sum of 5 Highest Values Difference parameters. Sum of the 5 Highest Values Frequence (Figure 4a) refers to the sum of the 5 highest partial optical density values for any given cell. This value provides an indication of the more dense areas of nucleic acid/protein (NA/P) composition of the cell and has been found to correlate closely with the mean frequency (mean optical density). A loss of correlation hypothetically could occur in cells with localized areas of high nucleic acid/protein concentration which might be expected to occur in rapidly synthesizing cells. For example, a cell of fairly uniform optical density could exhibit a sum of the 5 highest values of 210; or a cell could exhibit an area of concentrated nucleic acid/protein but also give a sum of 5 highest values of 210. The Sum of the 5 Highest Values Differences (Figure 4b) computes the sum of the 5 greatest differences between neighboring partial optical density values, giving an indication of nucleic acid/protein heterogeneity or

FIGURE 4

a. 5 HIGHEST VALUES



b. 5 HIGHEST VALUES DIFFERENCE



distribution. A homogeneous cell would give a small number for the sum of the 5 highest values difference between neighboring partial optical density values in contrast to a very heterogeneous cell which could display great variation from one partial optical density value to the next and provide a larger number for the sum of the 5 highest values difference.

In this manner much information was gained from individual lymphocytes within the population of lymphocytes. Simple statistical analyses were performed from the reduced data printouts and graphs of relative cell area versus total extinction (total optical density) were plotted.

Statistical Analyses

Student's t test as defined by Croxton (1953) was applied to scanning microscope photometry data to determine the significance of differences observed between (1) the responding and non-responding lymphocyte subpopulations following PWM stimulation and (2) the normal and the immunodeficient individuals. T numbers which gave a p value of .05 or less were considered significant.

The coefficient of correlation, two-variable linear formula, also explained by Croxton, was applied to determine the degree of correlation between the mean frequency and the Sum of the 5 Highest Values Frequency.

RESULTS

Immunologic Data

Plasma Immunoglobulin Levels

As stated previously, plasma immunoglobulin levels were determined for the IgG, IgA and IgM classes to demonstrate the striking contrast between the normal and severe combined immunodeficient subjects.

Table III shows the immunoglobulin values and normal ranges for the immunoglobulin classes analyzed.

Subject CC had normal values for the three plasma immunoglobulins, IgG, IgA and IgM. BW had a slightly elevated plasma IgM while SC and PC had slightly decreased IgA levels.

Subject DV, one of the severe combined immunodeficiency children, displayed low levels for all three immunoglobulins tested. Additional analyses performed in our laboratory showed that plasma levels of IgG on DV continued to decrease with time whereas the plasma IgM levels showed a possible slight increase, although remaining very low (unpublished data). The plasma IgA levels ranged from trace to undetectable. Analyses on DS also showed low levels of plasma IgG, IgA and IgM.

B Lymphocytes.

The percentage and absolute numbers of B lymphocyte subclasses were determined to aid in the assessment of immunologic response induced by pokeweed mitogen.

TABLE III

Plasma Immunoglobulin Levels

Category	Subject	Ig G mg % (770-1130)*	Ig A mg % (80-200)*	Ig M mg % (90-170)*
Normal	CC	1075	152	109
	BW	935	136	200
	SC	1100	64	137
	BB	ND	ND	ND
	PC	1005	61	174
SCID	DV 11/16/71 (Pre TF)	ND	ND	ND
	DV 11/22/71 (Pre TF)	292	Trace	10
	DV 6/29/73 (Post TF)	3	0	14
	DS 4/3/73	ND	ND	ND

SCID = Severe Combined Immunodeficiency Disease
ND = Not Done
TF = Transfer Factor

* Normal Range.

Table IV shows the lymphocyte data and normal ranges determined for our laboratory for the categories analyzed.

Subject CC gave a normal value for the per cent of total B lymphocytes. Subjects BW and PC gave a completely normal picture for B lymphocyte percentages and absolute numbers. Subject SC showed values for per cent and absolute numbers of B lymphocytes which were consistent with the low absolute lymphocyte count. In our laboratory, normal B lymphocyte per cent variation was found to be $\pm$ 2%. This would put the values within normal range.

No data was available on B lymphocytes for subjects BB or DS. Data was available on DV on 6/29/73; the data showed a high per cent of B lymphocytes. Additional studies performed on DV in our laboratory showed a decrease in the per cent of B lymphocytes with a concomitant shift in IgG-containing to IgM-containing cells with time (Criswell, personal communication)

Radioisotope Incorporation Rate Studies

Tritiated thymidine (^{3}H-TH) and tritiated uridine (^{3}H-UR) incorporation rates for measurement of DNA and RNA, respectively, were performed on the lymphocytes obtained from the normal individuals following pokeweed mitogen (PWM) stimulation. Table V shows the counts per minute (CPM) and stimulation indexes (SI) at 3, 5, and 7 days following PWM stimulation

TABLE IV

Lymphocyte Data

Category	Subject	Absolute Lymphocyte Count ($2.0-4.26 \times 10^3/mm^3$)	% B Lymphocytes (9-23)*	Absolute B Lymphocytes ($197-791/mm^3$)*	% G Lymphocytes (3-13)*	Absolute G Lymphocytes ($61-433/mm^3$)*	% A Lymphocytes (1-7)*	Absolute A Lymphocytes ($23-227/mm^3$)*	% M Lymphocytes (1-7)*	Absolute M Lymphocytes ($26-230/mm^3$)*
Normal	CC	ND	24	ND	ND	ND	ND	ND	ND	ND
	BW	2,496	9	225	3	75	2	50	5	125
	SC	1,608	7	113	5	80	2	32	0	0
	BB	2,862	ND	ND	ND	ND	ND	ND	ND	ND
	PC	2,380	17	405	7	167	3	71	7	167
	DV	ND	ND	ND	ND	ND	ND	ND	ND	ND
SCID	11/16/71 (Pre TF) DV	ND	57	ND	18	ND	7	ND	32	ND
	6/29/73 (Post TF) DS 4/3/73	5,814	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not Done
TF = Transfer Factor
SCID = Severe Combined Immunodeficiency Disease

* Normal Ranges.

TABLE V

^{3}H-TH and ^{3}H-UR Incorporation Values
for Normal Individuals

Subject	Day	^{3}H-TH Test CPM	^{3}H-TH Control CPM	^{3}H-TH Stimulation Index (T/C)	^{3}H-UR Test CPM	^{3}H-UR Control CPM	^{3}H-UR Stimulation Index (T/C)
CC	3 Day PWM	14,112	636	22	15,332	1,100	14
	5 Day PWM	43,373	1,193	36	16,258	1,193	14
	7 Day PWM	ND	ND	ND	ND	ND	ND
BW	3 Day PWM	34,407	2,379	15	15,077	2,123	6
	5 Day PWM	55,487	7,994	7	12,273	1,780	8
	7 Day PWM	41,574	6,965	6	9,618	1,274	8
SC	3 Day PWM	9,126	239	38	ND	ND	ND
	5 Day PWM	33,898	2,120	16	ND	ND	ND
	7 Day PWM	20,898	4,802	4	ND	ND	ND
BB	3 Day PWM	50,304	668	75	ND	ND	ND
	5 Day PWM	ND	ND	ND	ND	ND	ND
	7 Day PWM	ND	ND	ND	ND	ND	ND
PC	3 Day PWM	2,629	189	14	2,294	256	9
	5 Day PWM	7,509	1,088	7	4,441	798	6
	7 Day PWM	6,914	1,152	6	2,369	675	4

^{3}H-TH = Tritiated Thymidine
^{3}H-UR = Tritiated Uridine
CPM = Counts Per Minute
T/C = Test-Control
ND = Not Done

for tritiated thymidine and uridine incorporation rates. Counts per minute (CPM) values varied widely from individual to individual, but all samples showed a 4X or greater stimulation index, which was the criterion for positive stimulation, and which remained throughout the duration of the 7 day test period.

Based upon the CPM's, subjects SC and PC showed DNA synthesis occurring by day 3 with the peak DNA synthesis rate occurring at day 5 and decreasing by day 7. Subject CC showed the same pattern but was followed only at days 3 and 5. In contrast, subject BW displayed peak DNA synthesis at day 3, rather than day 5, also with a continuous decrease in the synthesis rate through day 7. Lymphocytes from BB indicated good stimulation at day 3 and were not subsequently followed, due to the quantity of blood required from a baby. Subjects BW and PC demonstrated peak RNA synthesis at day 3 whereas subject CC's synthesis rate remained stable from days 3-5.

Scanning Microscope Photometry Data

Normal Subjects

Normal Pokeweed Mitogen (PWM) Stimulated Cultures

Individual lymphocytes from subjects CC, BW and SC were analyzed by scanning microscope photometry at 3, 5 and 7 days following PWM stimulation for changes with respect to size and nucleic acid/protein content and heterogeneity. Graphs were plotted

of relative cell area versus total density (total O.D. or extinction).

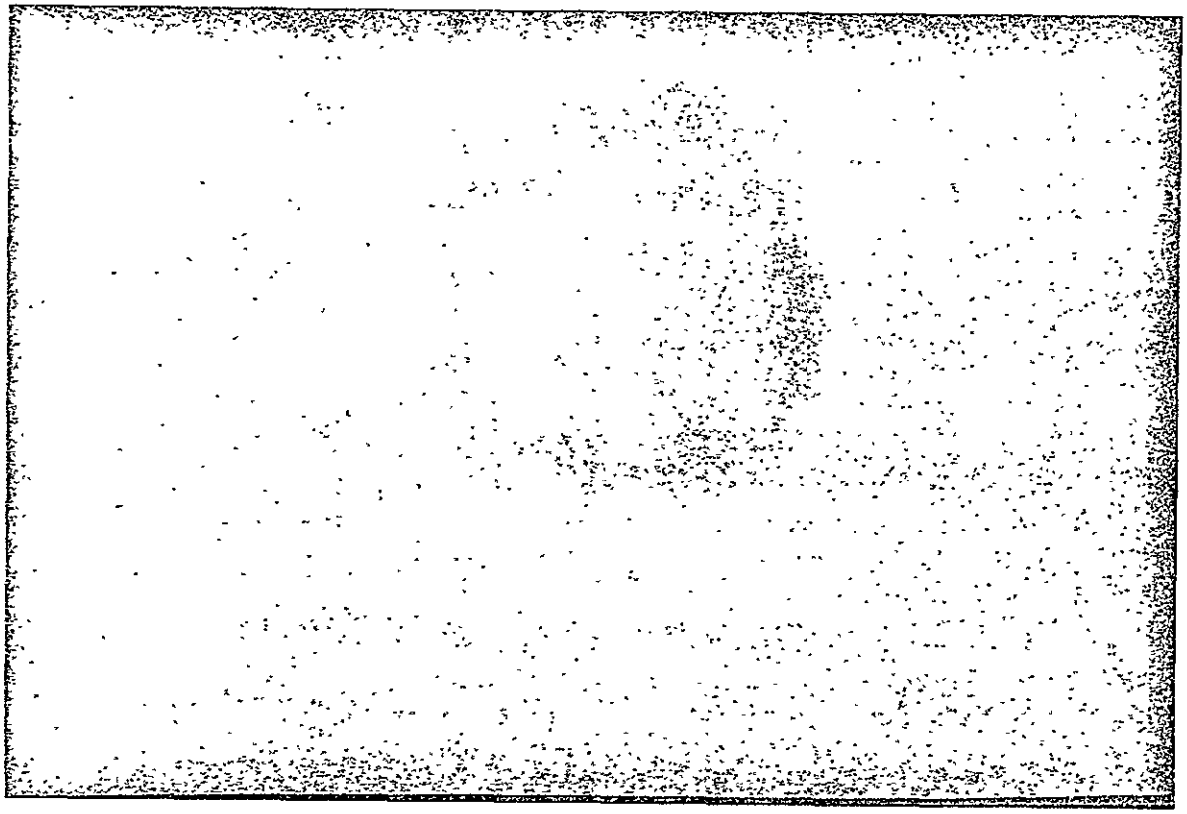
Two types of blastogenic cells were observed after 36 hours of PWM stimulation. Type I is thought to be derived from the T lymphocyte line while Type II is thought to be derived from the B lymphocyte line (Barker and Farnes, 1967, Chessin et al., 1967 and Douglas et al., 1967 and 1969).

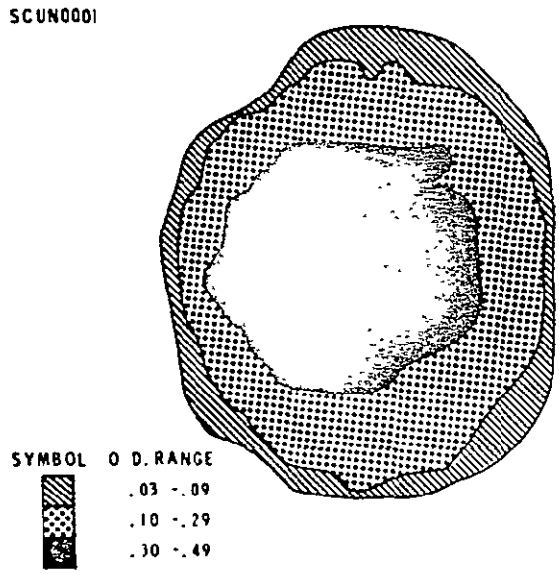
Figures 5-7 show pictures, graphic raw and reduced computer printouts of representative lymphocytes from subject SC. Figure 5 shows a picture of a representative unstimulated lymphocyte; 5b depicts a graphic representation of the raw printout for this cell and 5c gives the various parameters of the reduced printout for this cell. Similarly, Figure 6a-c shows a lymphocyte 5 days after PWM stimulation possibly of the lymphoblast type while figure 7a-c shows one possibly of the plasmablast type characterized by prominent nucleoli and cytoplasmic vacuoles.

Figure 8a-d shows graphs of relative cell area versus total density which illustrate the changes in the lymphocyte population of a representative subject, SC, at 0, 3, 5 and 7 days following PWM stimulation. Dotted circles show the comparison of the peripheral blood unstimulated lymphocytes to the PWM stimulated lymphocyte populations. An appreciable number of cells fell outside the boundaries of the unstimulated samples with respect to relative cell area and total density for all three normal

FIGURE 5a. Representative Unstimulated Lymphocyte from Subject SC.

FIGURE 5b. Graphic Representation of Computer Raw Printout for This Cell



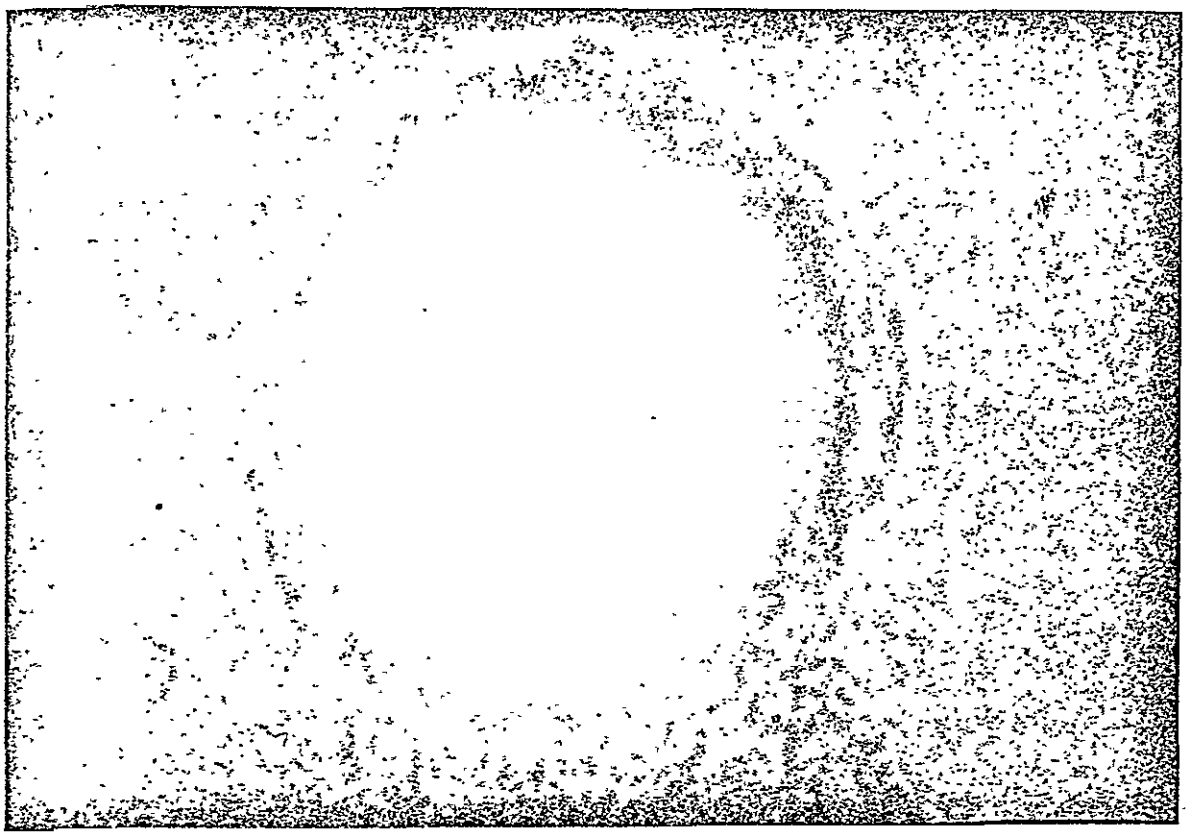

SCUN0001
SYMBOL O D. RANGE
.03 -.09
.10 -.29
.30 -.49

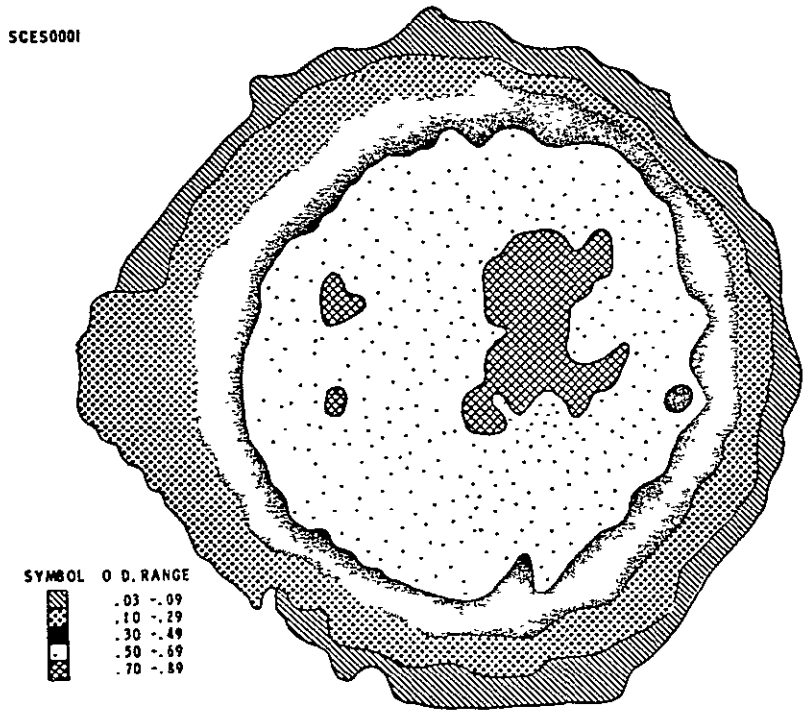
```
NAME

AREA  225
TOTAL EXTINCTION   +5.014000E+003
MEAN FREQUENCY    22
MEDIAN FREQUENCY     23
MEAN DIFFERENCE       4
MEDIAN DIFFERENCE         4
SUM OF    5 HIGHEST VALUES FREQUENCY  215
SUM OF   10 HIGHEST VALUES FREQUENCY  420
SUM OF    5 HIGHEST VALUES DIFFERENCE     82
SUM OF   10 HIGHEST VALUES DIFFERENCE    138
```

FIGURE 5c. Computer Reduced Printout for This Cell.

FIGURE 6a. Possible Lymphoblast from Subject SC 5 Days after PWM Stimulation.

FIGURE 6b. Graphic Representation of Computer Raw Printout for This Cell.



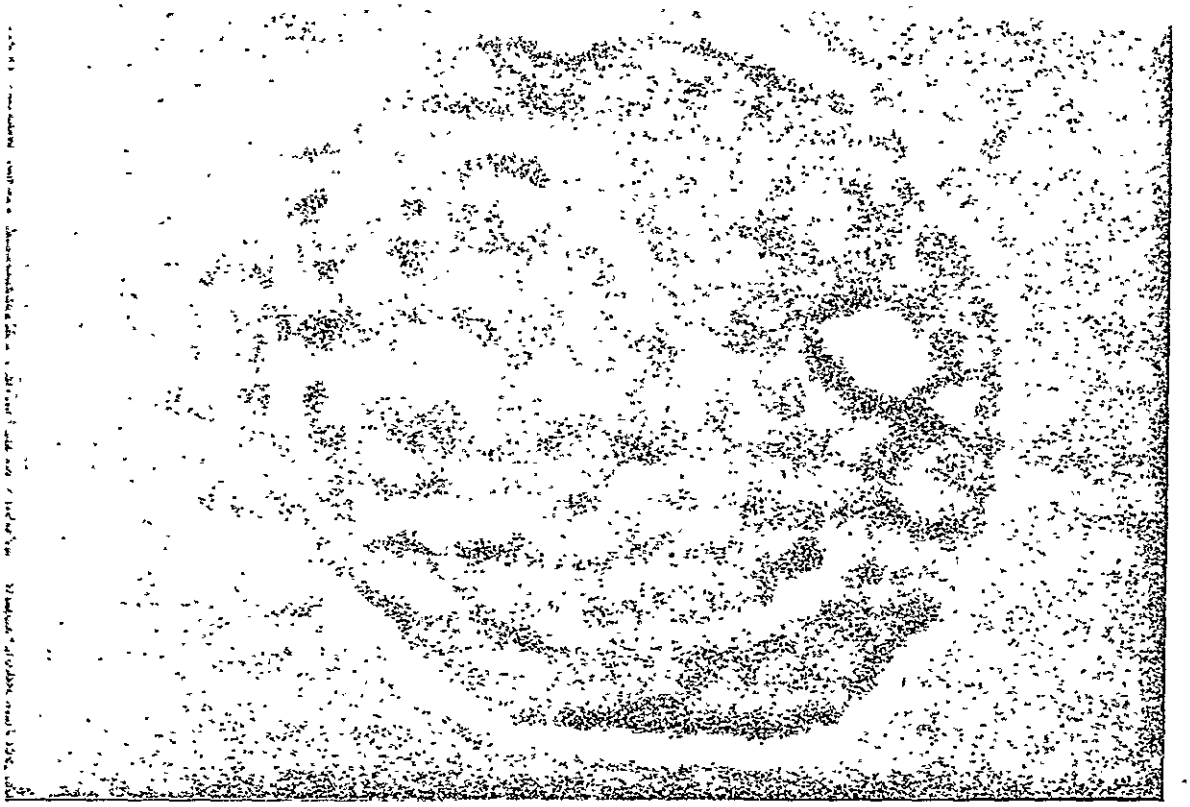

SCES0001
SYMBOL
O D. RANGE
.03 -.09
.10 -.29
.30 -.49
.50 -.69
.70 -.89

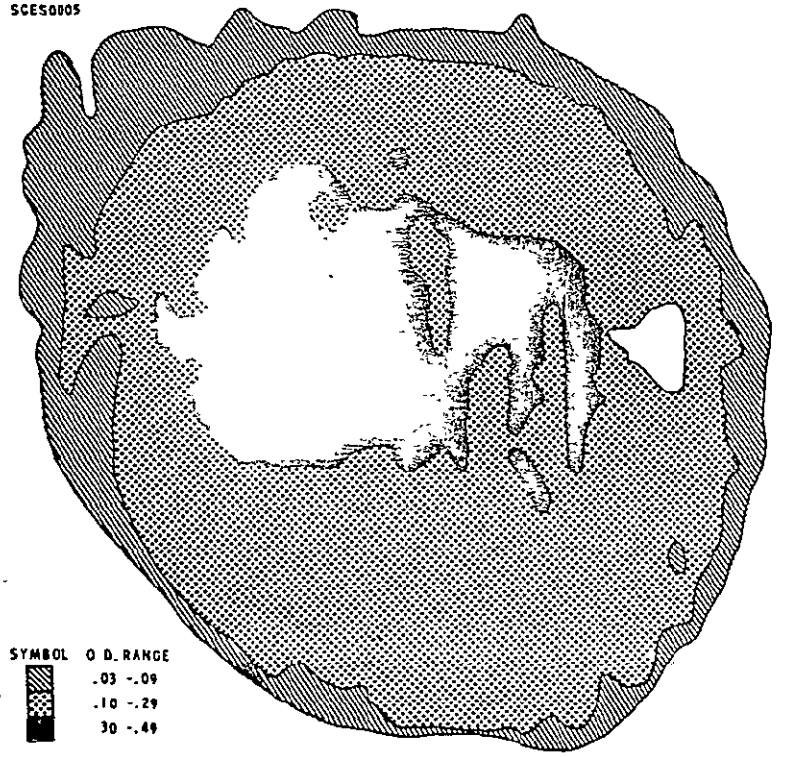
```
NAME

AREA  756
TOTAL EXTINCTION    +3.165800E+004
MEAN FREQUENCY     41
MEDIAN FREQUENCY     48
MEAN DIFFERENCE       5
MEDIAN DIFFERENCE        5
SUM OF    5 HIGHEST VALUES FREQUENCY  385
SUM OF   10 HIGHEST VALUES FREQUENCY  755
SUM OF    5 HIGHEST VALUES DIFFERENCE    128
SUM OF   10 HIGHEST VALUES DIFFERENCE    236
```

FIGURE 6c. Computer Reduced Printout for This Cell

FIGURE 7a. Possible Plasmablast from Subject SC 5 days After PWM Stimulation.

FIGURE 7b. Graphic Representation of Computer Raw Printout for This Cell.




SCES0005
SYMBOL
O D. RANGE
.03 -.09
.10 -.29
.30 -.49

```
NAME

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MEDIAN FREQUENCY   20
MEAN DIFFERENCE     3
MEDIAN DIFFERENCE      4
SUM OF    5 HIGHEST VALUES FREQUENCY  226
SUM OF   10 HIGHEST VALUES FREQUENCY  437
SUM OF    5 HIGHEST VALUES DIFFERENCE     87
SUM OF   10 HIGHEST VALUES DIFFERENCE    153
```
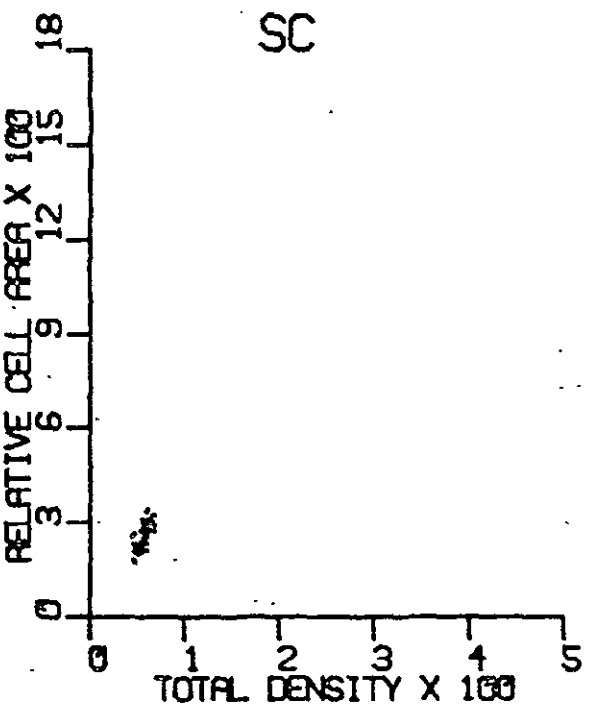
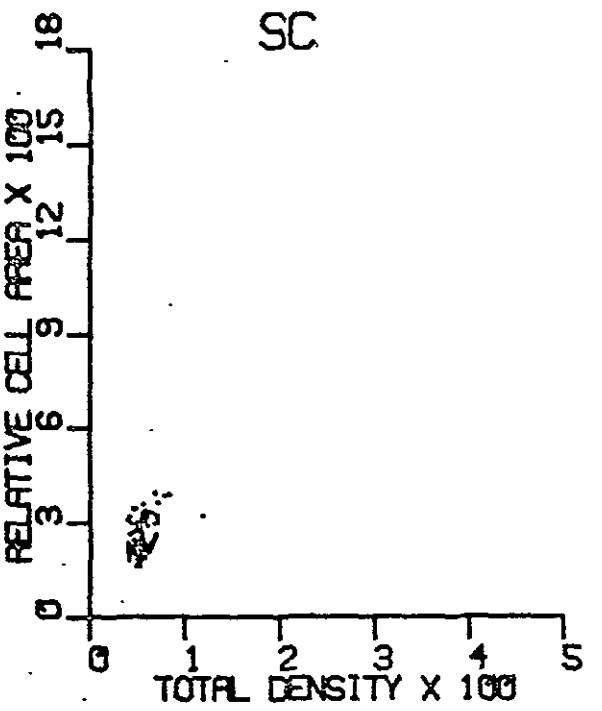

FIGURE 7c. Computer Reduced Printout for This Cell

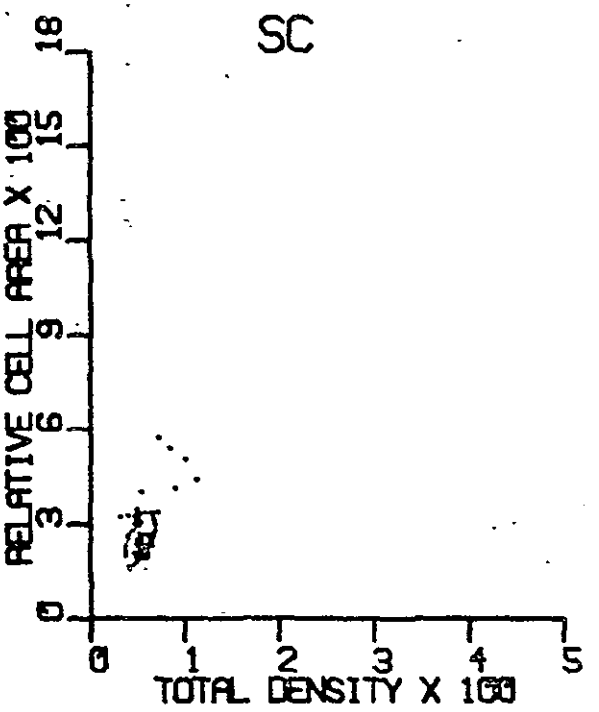
FIGURE 8



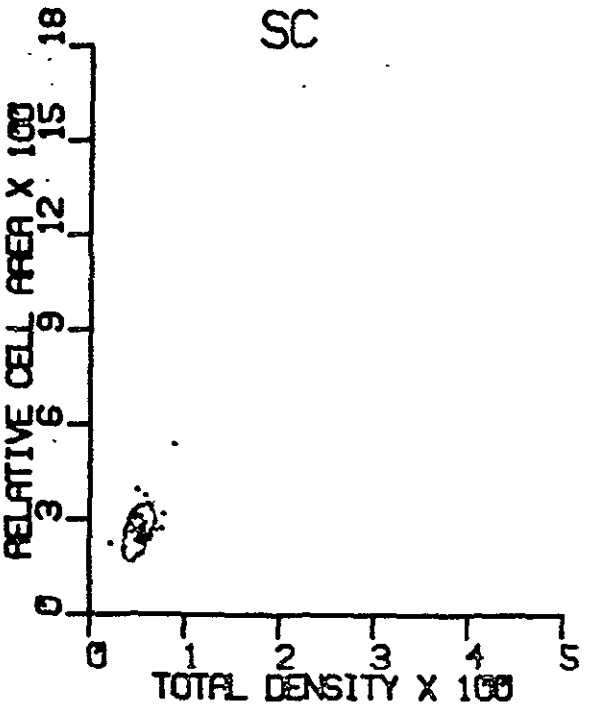
a Subject SC Unstimulated Lymphocytes



b Subject SC 3 Day PWM Stimulated Lymphocytes



c Subject SC 5 Day PWM Stimulated Lymphocytes



d Subject SC 7 Day PWM Stimulated Lymphocytes

subjects. These cells were classified as "responding" while the remainder were termed "non-responding". They varied from 8-42% of the lymphocyte population among the three individuals.

Tables VI, VII and VIII show the scanning microscope photometry values obtained for the lymphocyte subpopulations from subjects CC, BW and SC, respectively. Student's t tests were applied to the responding and non-responding subpopulation values to determine differences significant at the 95% confidence level or greater. (Croxton, 1953).

Subject CC, Table VI, showed maximal response to PWM at day 5 in terms of relative cell area, total density and 5 highest values difference parameters. The mean frequency and 5 highest values continued to increase throughout day 7. This finding indicated that some synthesis was still occurring, possibly due to repeated division of an initially small population of cells responsive to PWM and/or due to a population of cells not as rapidly activated by PWM (Weber, 1973). Subject CC also showed no significant difference in the 5 highest values difference parameter at day 7, suggesting a decline in nucleic acid/protein synthesis. The coefficient of correlation test between the mean frequency and 5 highest values parameters showed no loss of correlation during the 3-7 day test period.

TABLE VI

Subject CC SMP Response Curve Values

Day	n	Per Cent	Diameter (μ)	Relative Cell Area	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
Unstimulated	51	--	7.7	185 ± 32	55 ± 9	30 ± 5	278 ± 35	113 ± 29
3 Day PWM								
NR	49	89	8.4	219 ± 34**	49 ± 6**	22 ± 3**	210 ± 39**	86 ± 12*
R	6	11	12.4	481 ±217	92 ± 63	18 ± 4	161 ± 33	73 ± 20
5 Day PWM								
NR	36	74	8.7	238 ± 45**	51 ± 28**	21 ± 4**	180 ± 38	76 ± 17**
R	13	27	14.4	652 ±327	161 ± 75	25 ± 5	200 ± 36	127 ± 14
7 Day PWM								
NR	35	66	7.8	191 ± 34**	55 ± 6**	29 ± 6*	266 ± 46**	106 ± 28
R	18	34	13.5	573 ±284	154 ± 93	26 ± 5	221 ± 41	110 ± 47

NR = Non-responding
R = Responding
* $P < .05$
** $P < .01$
n = number per category

TABLE VII

Subject BW SMP Response Curve Values

Day	n	Per Cent	Diameter (μ)	Relative Cell Area	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
Unstimulated	57	--	8.6	230 ± 44	51 ± 8	22 ± 3	200 ± 36	85 ± 16
3 Day PWM								
NR	47	92	9.0	257 ± 49**	47 ± 8**	18 ± 3	158 ± 30	65 ± 14
R	4	8	12.3	475 ±146	84 ±30	17 ± 3	149 ± 39	63 ± 13
5 Day PWM								
NR	36	71	8.5	228 ± 34**	47 ± 7**	20 ± 3	171 ± 25*	74 ± 13**
R	15	29	13.2	550 ±185	121 ±49	21 ± 2 Δ	191 ± 45	106 ± 38
7 Day PWM								
NR	38	75	8.8	242 ± 54**	51 ± 8**	21 ± 4	186 ± 32	80 ± 21
R	13	26	13.6	581 ±147	123 ±43	21 ± 4	176 ± 30	88 ± 33

NR = Non-responding
R = Responding
* $P < .05$
** $P < .01$
n = number per category
Δ = loss of correlation

TABLE VIII

Subject SC SMP Response Curve Values

Day	n	Per Cent	Diameter (μ)	Relative Cell Area	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
Unstimulated	58	--	9.0	256 ± 43	56 ± 7	22 ± 3	232 ± 31	76 ± 14
3 Day PWM								
NR	41	72	9.0	257 ± 50**	51 ± 6**	20 ± 5*	187 ± 45	80 ± 22*
R	16	28	14.1	628 ±260	160 ±97	25 ± 8	207 ± 59	104 ± 44
5 Day PWM								
NR	33	60	9.2	263 ± 50**	55 ± 8**	21 ± 5*	195 ± 50	69 ± 23**
R	22	40	15.3	738 ±223	191 ±87	25 ± 8	221 ± 65	100 ± 49
7 Day PWM								
NR	30	58	9.5	281 ± 49**	54 ± 10**	19 ± 5**	162 ± 38**	64 ± 11**
R	21	42	14.4	647 ±162	150 ± 43	23 ± 4	207 ± 45	94 ± 51

NR = Non-responding
R = Responding
* $P < .05$
** $P < .01$
n = number per category

Subject BW, Table VII, showed increases in both relative cell area and total density values through day 7. The 5 highest values and 5 highest values difference parameters peaked at day 5 and decreased by day 7; this suggested that although nucleic acid/protein synthesis was still occurring through day 7, peak synthesis was most active at day 5. In addition, a loss of correlation was noted between the mean frequency and 5 highest values parameters at day 5, indicating the presence of areas of concentrated nucleic acid/protein, suggestive of rapidly synthesizing cells. Student's t test confirmed a significant difference in the 5 highest values difference parameter between the responding and non-responding subpopulations only at day 5, lending further support to the observation that peak response to PWM occurred at day 5. These findings suggested the presence of concentrated areas of nucleic acid/protein in a heterogeneous distribution. Mean frequencies never became significantly different during the 3-7 day period, implying proportional changes in cell size and nucleic acid/protein content.

Subject SC, Table VIII, was similar to subject CC in that SC also showed peak response to PWM at day 5 with respect to relative cell area, total density and 5 highest values. The mean frequency, however, remained the same at days 3 and 5, indicating proportional changes in cell size and nucleic acid/protein content, but dropped at day 7, suggesting a tapering-off of nucleic acid/protein synthesis. It was

found that the mean frequency and 5 highest values difference parameters were significantly different at days 3, 5 and 7. The fact that the 5 highest values parameters were not significantly different on these days suggested that, although a great amount of synthesis was occurring, there were no localized areas of concentrated nucleic acid/protein. This was supported by the coefficient of correlation test which showed no loss of correlation between the mean frequency and 5 highest values parameters.

Table IX shows the mean SMP values for the three normal individuals at 0, 3, 5 and 7 days. Among the responding cell populations, relative cell area, total density and 5 highest values difference parameters were significantly increased by day 3 and showed peak response at day 5. This was supported by the 5 highest values and mean frequency parameters which also peaked and were only significantly different at day 5, lending further support to maximal response to PWM stimulation at day 5.

By using scanning microscope photometry to observe changes in cell size, nucleic acid/protein content, synthesis and heterogeneity, it can be stated that blastogenesis due to PWM stimulation was quite apparent by day 3 with peak blastogenic response occurring at day 5 and tapering off by day 7.

Normal Non-Stimulated Cultures

Figure 9 shows graphs with respect to relative cell area and total density for the unstimulated peripheral blood and non-stimulated lymphocytes cultured and harvested at 3, 5 and 7 days.

TABLE IX

Mean SMP Values Following PWM Stimulation

Day	n	Per Cent	Diameter (μ)	Relative Cell Area	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
Unstimulated	166	--	8.5	226 ± 40	54 ± 8	25 ± 4	235 ± 34	91 ± 20
3 Day PWM								
NR	137	84	8.8	243 ± 44**	49 ± 7**	20 ± 4	185 ± 30	77 ± 16**
R	26	16	13.5	572 ±225	132 ±79	22 ± 6	188 ± 50	91 ± 35
5 Day PWM								
NR	105	68	8.8	242 ± 43**	51 ± 8**	21 ± 4**	182 ± 38**	73 ± 18**
R	50	32	14.5	659 ±241	162 ±72	24 ± 6	207 ± 52	109 ± 43
7 Day PWM								
NR	103	66	8.7	236 ± 46**	53 ± 8**	23 ± 5	206 ± 38	84 ± 21*
R	52	34	13.9	605 ±204	145 ±63	24 ± 4	204 ± 39	98 ± 44

NR = Non-responding

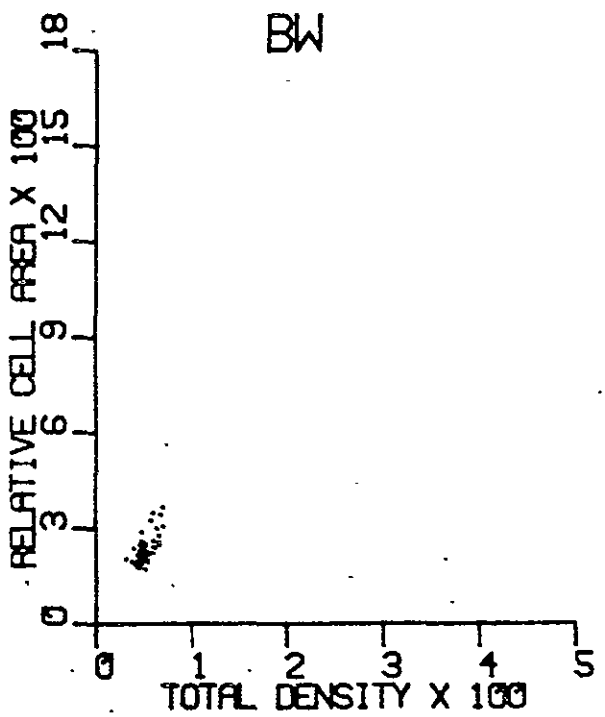
R = Responding

* $P < .05$

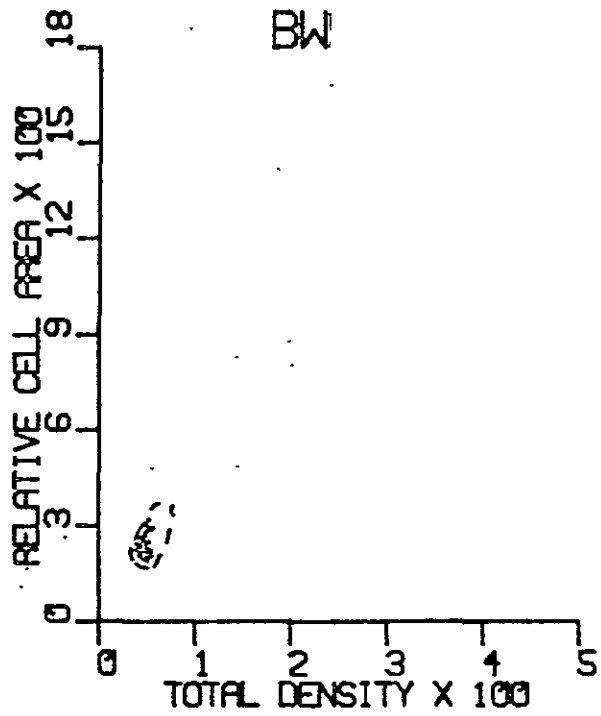
** $P < .001$

n = number per category

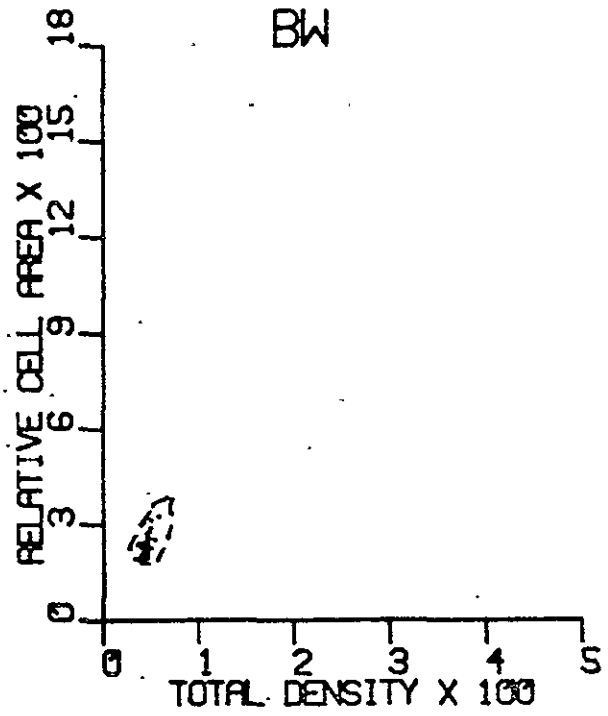
FIGURE 9



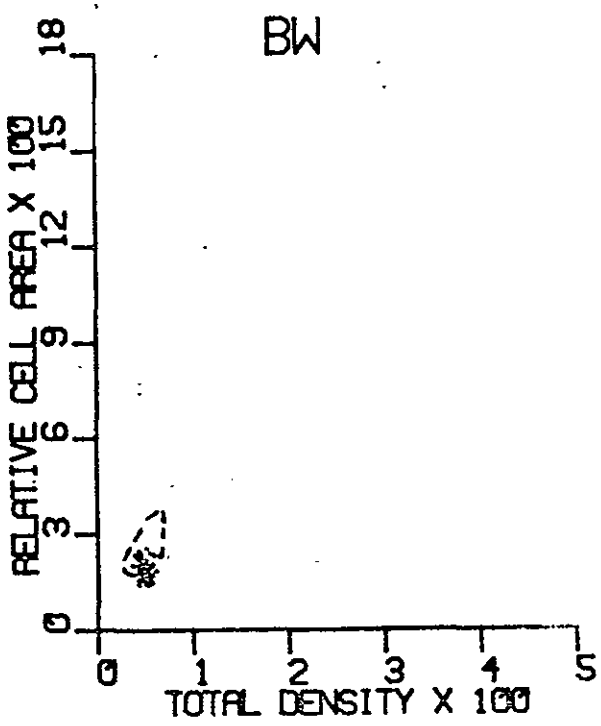
a Unstimulated Lymphocytes



b Non-stimulated Cultured Lymphocytes at 3 days.



c Non-stimulated Cultured Lymphocytes at 5 days



d Non-stimulated Cultured Lymphocytes at 7 days

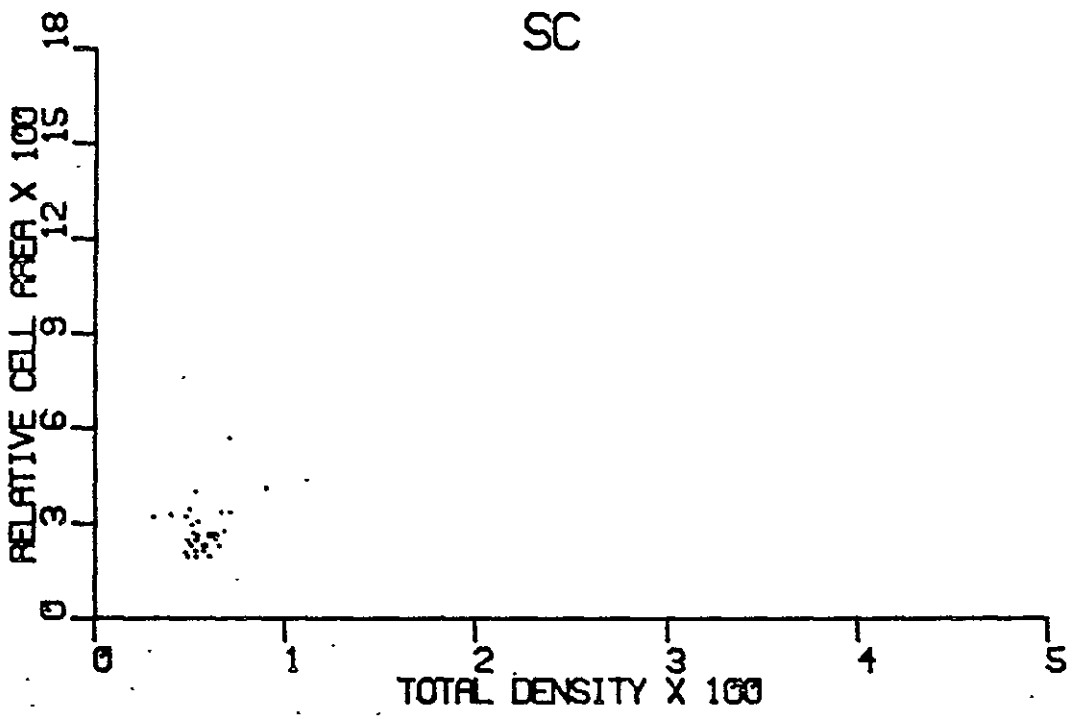
Comparison of the unstimulated and the non-stimulated cultured lymphocytes basically showed little change during the 7 day test period.

Slight changes were noted in a slight decrease in total density at day 3, resulting in a slightly decreased mean frequency. At day 5 the cells showed a slight decrease in relative cell area, possibly due to equilibration in the tissue culture environment. At day 7 the cells appeared slightly smaller and more dense, possibly due to a change in the cell cycle phase. Therefore, to negate any changes in lymphocytes due to tissue culture environment, responding and non-responding subpopulations within the PWM stimulated cultures were compared to each other rather than to the peripheral blood unstimulated or nonstimulated cultured lymphocytes.

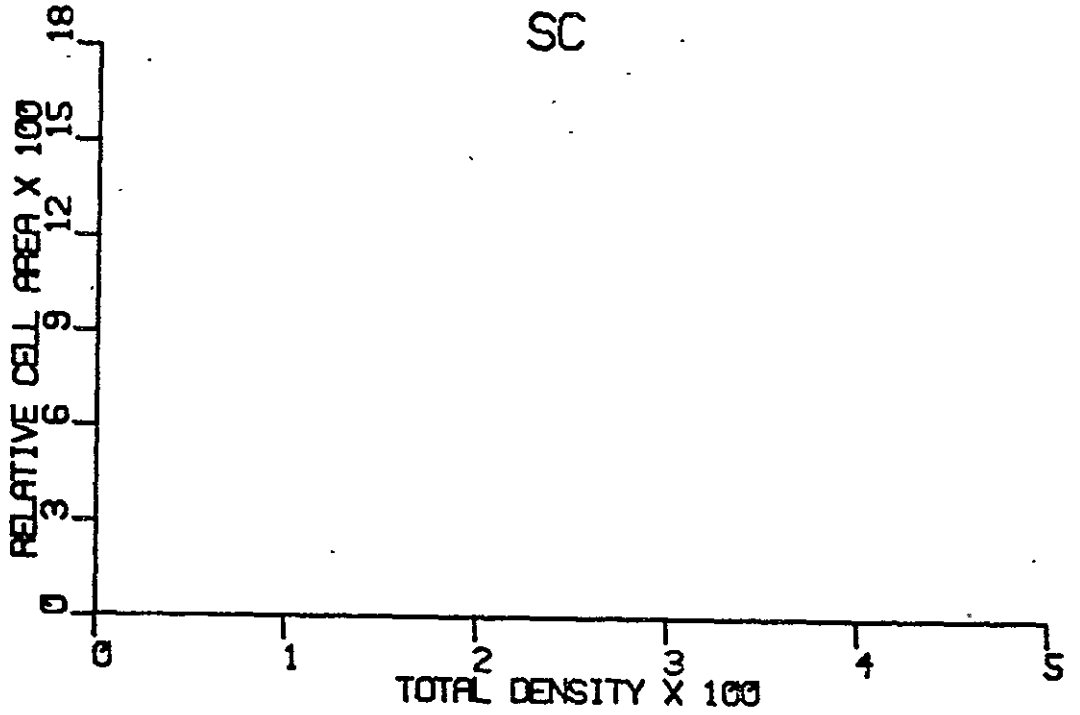
Sample Biasing

Lymphocytes were normally selected at random but an experiment was performed to determine the effects of sample biasing. Lymphocytes after 5 days PWM stimulation were scanned first in random fashion and then by selecting only those cells which were obviously undergoing blastogenesis. Figure 10 shows two graphs comparing the 5 day "random" and "blastogenic" lymphocytes with respect to relative cell area and total density (extinction). The randomly-selected population displays many smaller lymphocytes. Table X shows the scanning microscope photometry values for the "random" and "blastogenic"

FIGURE 10



a Effects of Sample Biasing. "Random" Cell Selection.



b Effects of Sample Biasing. "Blastogenic" Cell Selection

C-2

TABLE X

SMP Values for "Random" Versus

"Blastogenic" Lymphocytes

	n	Relative Cell Area	Diameter (μ)	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
Random	55	453 ± 276	12.0	109 ± 87	23 ± 6	206 ± 58	81 ± 38
		**		**	*	**	*
Blastogenic	33	765 ± 232	15.6	213 ± 81	27 ± 7	251 ± 60	106 ± 44

* $P < .02$

** $P < .001$

n = number per category

lymphocytes. Obvious differences were shown with respect to all parameters at a confidence level of 98 per cent or greater. We elected to use the random scan method in order to obtain quantitative, rather than just qualitative, results.

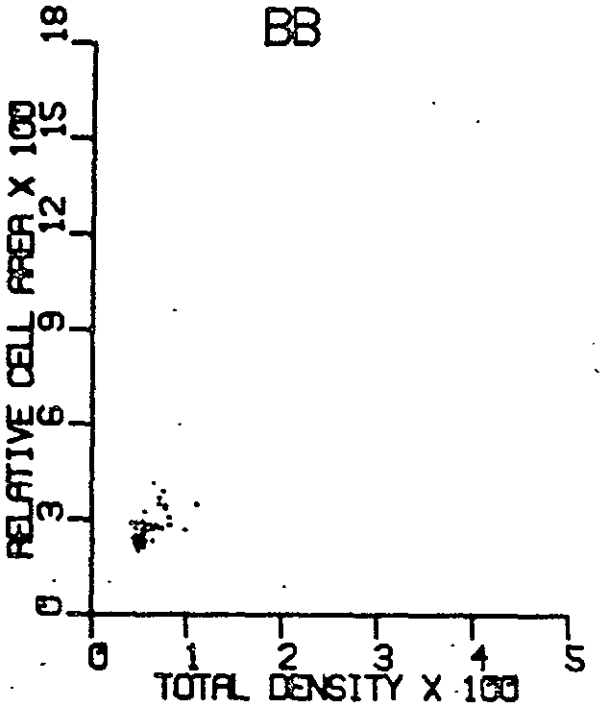
Immunodeficient Subjects

Lymphocytes from two subjects with severe combined immunodeficiency disease (SCID) were examined by scanning microscope photometry and compared to five normals to obtain information which may prove useful in better understanding the basic pathologic defects.

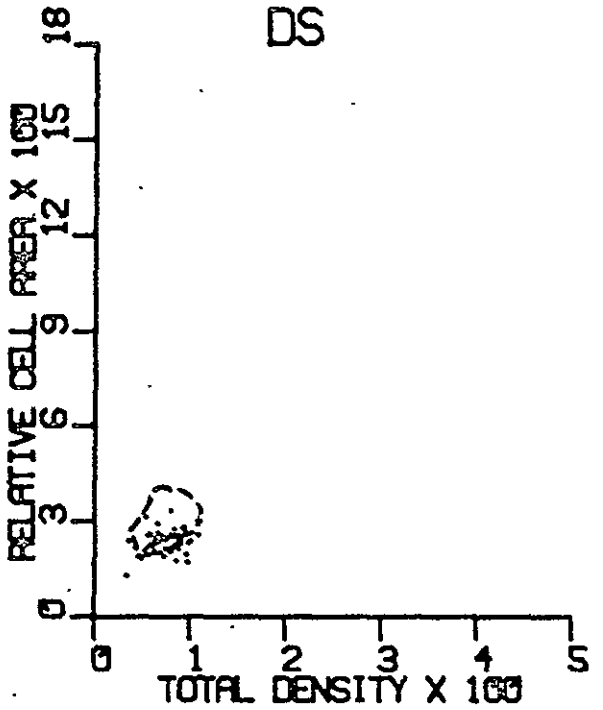
Graphs of relative cell area versus total density were plotted for the two immunodeficient subjects, DS and DV, and compared to the normal baby, BB, in Figure 11. Figure 11a depicts the lymphocyte pattern for the normal baby; 11b shows the pattern for subject DS while 11c and d show the lymphocyte patterns for subject DV before and after treatment with transfer factor. Lymphocytes from DS appeared denser than normal while those from DV appeared less dense than normal before the administration of transfer factor and more dense after transfer factor, approaching a more normal pattern.

Table XI shows the SMP values of the five normal and the two immunodeficient subjects. Student's t tests were used to compare (1) the immunodeficient to the mean normal values and (2) DVvalues before and after the administration of transfer factor.

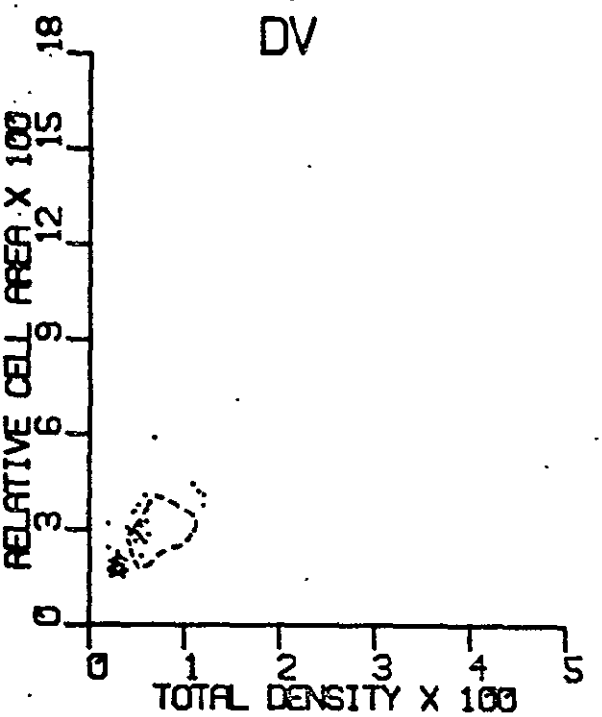
FIGURE 11



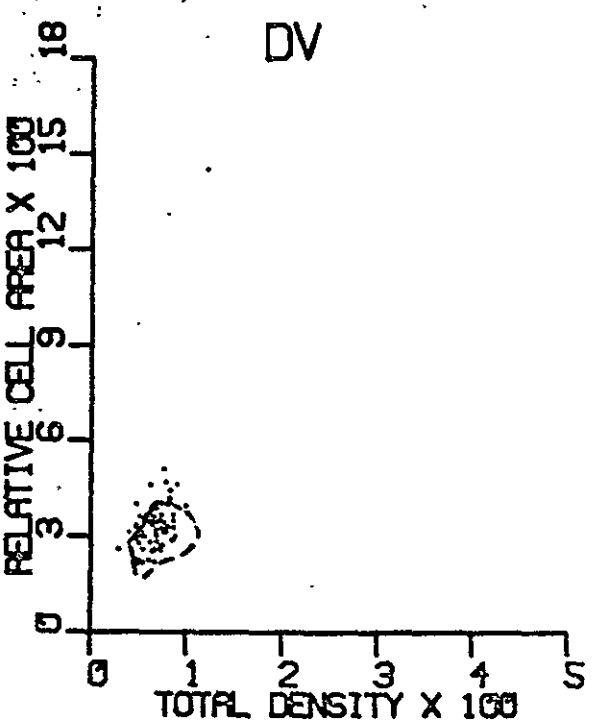
a Lymphocytes from a Normal Baby



b Lymphocytes from an Immuno-deficient Baby



c Lymphocytes from an Immuno-deficient Baby Before Treatment with Transfer Factor



d Lymphocytes from an Immuno-deficient Baby after Treatment with Transfer Factor.

TABLE XI

Comparison of Normal and Immunodeficient SMP Values

Subject	n	Diameter (μ)	Relative Cell Area	Total Density (Extinction)	Mean Frequency	5 Highest Values	5 Highest Values Difference
CC	51		185 ± 32	55 ± 9	30 ± 5	278 ± 35	113 ± 29
BW	57		230 ± 38	51 ± 8	22 ± 3	200 ± 36	85 ± 16
SC	58		256 ± 43	56 ± 7	22 ± 3	232 ± 31	76 ± 14
PC	57		223 ± 39	54 ± 7	24 ± 4	229 ± 43	80 ± 17
BB	56		263 ± 48	58 ±14	22 ± 4	195 ± 38	70 ± 24
	279	8.6	233 ± 40	55 ± 9	24 ± 4	226 ± 36	85 ± 20
DS	48	8.7	238 ± 48	81 ±24**	34 ±10**	342 ±102**	143 ± 56**
DV 11/16/71 (Pre TF)	49	9.0	252 ± 97* ††	43 ±25 ††	16 ± 5** ††	163 ± 62** ††	93 ± 28* †
DV 6/29/73 (Post TF)	57	10.3	330 ± 71**	67 ±14**	20 ± 5**	189 ± 52**	81 ± 31

Normals: CC, BW, SC, PC, BB

Immunodeficients: DS, DV

TF = Transfer Factor

* P < .05 to normal mean

** P < .001 to normal mean

† P< .05 pre TF to post TF

†† P< .001 pre TF to post TF

Subject DS demonstrated lymphocytes that were normal in size but very high in nucleic acid/protein content and heterogeneous in nucleic acid/protein distribution, as reflected by the total density and the 5 highest values difference parameters. The cells were denser than normal as suggested by the mean frequency and 5 highest values parameters. Student's t test confirmed that total density, mean frequency, 5 highest values and 5 highest values difference parameters were significantly different from the normal population at the 99.9% confidence level. This suggested very active cells synthesizing some type(s) of product(s), the biochemical nature of which is/are undetermined.

Lymphocytes from subject DV were analyzed by scanning microscope photometry nine months before and after multiple injections of transfer factor. At approximately one month of age on 11/16/71, DV appeared to have two types of lymphocytes. The majority of the cells (76%) were small lymphocytes with a vacuolated, "punched-out" chromatin appearance while fewer in number (24%) were large lymphocytes with a more uniform chromatin pattern that appeared "balloony". Overall, the data showed lymphocytes slightly larger and less dense than normal, as reflected by the relative cell area, mean frequency and 5 highest values parameters. The 5 highest values difference parameter was in agreement with the more heterogeneous chromatin appearance.

On 6/29/73, nine months after the administration of transfer factor, DV displayed a shift in lymphocyte populations. The majority of the lymphocytes (79%) were larger with a higher nucleic acid/protein content than normal, as reflected by the relative cell area and total density values. Proportionally, however, the mean frequency was significantly lower than the value for the normal lymphocytes, which was supported by the 5 highest values parameter. The heterogeneity, or distribution, of the nucleic acid/protein was not significantly different from the normal population, as reflected by the 5 highest values difference parameter.

Student's tests were applied to the pre- and post-transfer factor SMP values. They confirmed that all parameters had changed significantly at or above the 95% confidence level with respect to relative cell area, total density, mean frequency, 5 highest values and 5 highest values difference parameters. It appeared, therefore, that after treatment with transfer factor, the lymphocyte population shifted from many small, sparse lymphocytes with a heterogeneous chromatin pattern to lymphocytes that fell into a more normal pattern displaying a more normal chromatin appearance and an increase in nucleic acid/protein although they had become even larger in size, resulting in a mean frequency lower than normal.

Clinical studies also indicated that a change had taken place within the lymphocyte subpopulations. Lymphocytes from DV at 3-10 months of age showed that 90% of the cells contained membrane-bound immuno-

globulins. These cells did not respond to in vitro stimuli which included phytohemagglutinin (PHA), PWM, keyhole limpet hemocyanin (KLH), streptokinase-streptodornase (SK-SD), purified protein derivative (PPD), Candida and allogenic cells. After the administration of transfer factor obtained from a donor sensitive to PPD, SK-SD and Candida, subject DV gave positive skin tests to KLH, SK-SD and Candida, indicating that delayed hypersensitivity had been transferred. This reaction, however, was transitory and disappeared after 2-3 injections of transfer factor. Additional injections of transfer factor produced no change and the lymphocytes still did not respond to in vitro stimuli. Four months after transfer factor 90% of the lymphocytes still contained membrane-bound immunoglobulins; twelve months after transfer factor, 60% of the lymphocytes were positive for membrane-bound immunoglobulin (B cells) and 3-7% were positive for the E rosette test (T cells). These findings indicated that transfer factor possibly aided in the maturation of the T lymphocyte population and/or caused structural changes in the B lymphocyte population (Criswell et al., 1974). Scanning microscope photometry proved useful in providing a quantitative assessment of the degree of structural change.

DISCUSSION

Lymphocytes were obtained from normal subjects, stimulated with pokeweed mitogen (PWM) and analyzed by scanning microscope photometry (SMP) to obtain detailed information concerning cellular structure not discernable by the naked eye. This information was used to : (1) construct a normal blastogenic response curve to aid in a better understanding of the basic events occurring during a normal immune response. In addition, the normal nonstimulated lymphocytes were used as baseline normals and compared to lymphocytes from patients with immune deficiency disease.

An individual shown to display a deviant blastogenic response curve may be said to evoke an abnormal immune response which may possibly result from a defect(s) at various levels such as: (1) at the level of recognition, possibly due to defective receptor sites on the lymphocyte membrane, causing a lack of response, (2) at the level of transcription, causing asynchronous changes in cell size and nucleic acid/protein synthesis or (3) at the level of translation, causing production of a defective product(s) such as an enzyme or incomplete immunoglobulin.

Non-specific mitogenic stimulation was shown by Greaves and Janossy (1972) to mimic antigenic stimulation in the elicitation of B and T lymphocyte responses. Pokeweed mitogen was shown to induce blastoid transformation, or blastogenesis, in PWM-responsive lymphocytes by first attaching to binding sites located on the carbohydrate moiety

of the lymphocyte surface membrane (Greaves and Janossy, 1971 and van der Berg and Betel, 1974) and then activating the lymphocytes by a process as yet unknown but postulated to involve the generation of a triggering signal at the level of the surface membrane. According to Bartels and Wied (1969),

> the gross changes in the total amounts of cytochemical constituents of lymphocytes undergoing transformation can be expected to be accompanied by organizational changes which should express themselves in the data structure of digitized optical images of such cells.

Blastogenic transformation was indeed observed using scanning microscope photometry. This technique obtained data which detected trends indicative of an immune response by providing information relating to changes in cell size and nucleic acid/protein content and heterogeneity. Changes were detected by day 3 following PWM stimulation, with peak blastogenic response shown to occur at day 5 and decreased by day 7.

The SMP data presented here was obtained by taking optical density measurements of randomly-selected lymphocytes at 0.5 μ intervals across a cell at a scanning wavelength of 280 nm and an optical density threshold above 0.02. The data of Bartels and Wied (1969) was obtained on pre-selected blastogenic lymphocytes by taking optical density measurements at 260 nm and 280 nm, with an optical density threshold of 0.03. Their data showed highly significant changes in Phytohemagglutinin-stimulated (PHA) lymphocytes compared to unstimulated lymphocytes. An experiment was performed in our laboratory to determine the differences in results

obtained by random versus blastogenic cell selection (see Section "Sample Biasing", p. 91). This data showed significant differences in all SMP parameters at the 98-99.9% confidence levels. Therefore, the decision was made to obtain our data on random rather than pre-selected blastogenic lymphocytes in order to obtain a quantitative measurement of the degree of blastogenesis occurring in vitro, representative of an in vivo immune response (Greaves and Janossy, 1972).

Our data showed the same trends following mitogenic stimulation as did Bartels et al. (1969) in terms of increases in relative cell area and total density (extinction), but our peak values differed as to the time after stimulation. These differences may be explained by the fact that they used PHA instead of PWM. PHA is known to stimulate primarily T lymphocytes (Janossy and Greaves, 1972), causing up to 80% to become transformed whereas pokeweed mitogen has been shown to stimulate both B and mature T lymphocytes (Greaves and Janossy, 1972) causing only 40-60% of the lymphocytes to undergo blastogenesis (Douglas, 1972). In this study, it was shown that up to 42% of the lymphocytes did undergo blastogenesis due to PWM and that the percentage increased with respect to time in culture. It has been postulated that the increase in the number of lymphocytes responding to PWM with time may be due to a continual or prolonged period of cell recruitment, to repeated division of an initially small population of PWM-responsive B lymphocytes, or that B lymphocytes

are not activated by PWM as rapidly as T lymphocytes (Weber, 1973). However, our data comparing the percentage of B lymphocytes in the peripheral blood to the number of lymphocytes responding to in vitro PWM stimulation proved to be inconclusive. Reports by various investigators (Barker and Farnes, 1967, Chessin et al., 1967 and Douglas et al., 1967 and 1969) stated that two types of blast lymphocytes may be observed after PWM stimulation. Type I, termed an immunoblast, is a large blast cell with cytoplasmic vacuoles, identical to those seen in PHA-stimulated cultures, while Type II, termed a plasmablast, is unique to PWM and generally possesses multiple nucleoli and an eccentric nucleus. Type II cells may not be found before 36 hours of stimulation and show a loose chromatin pattern by 72 hours. According to Douglas (1972), approximately 25% of the blast cells are Type II. Two types of blast cells were observed by scanning microscope photometry but were not defined as to their origin or frequency of occurrence.

This study showed that scanning microscope photometry was sensitive enough to provide quantitative information about individual lymphocytes within a population with respect to changes in cell size and nucleic acid/protein content and heterogeneity after mitogenic stimulation. The fact that significant trends were demonstrated by day 3 leads one to suspect that elicitation of a normal blastogenic response curve may be demonstrated at an earlier time period.

The data obtained by scanning microscope photometry was augmented with measurement of DNA and RNA synthesis rates to ascertain, to a

greater extent, the effects of blastogenesis upon biochemical and morphological changes. As stated previously, maximal blastogenic response to pokeweed mitogen was demonstrated at day 5 by scanning microscope photometry. Radioisotope incorporation data showed peak DNA synthesis at day 3 for one of the three normal individuals. This finding was consistent with the studies of Douglas et al. (1966) and Chessin et al. (1966) which showed peak DNA synthesis from 66-72 hours preceded by peak RNA synthesis 24 hours earlier. The other two normal subjects showed peak DNA synthesis at day 5. This finding was also reported by Douglas et al. (1969). Peak RNA synthesis was found by day 3, decreasing through day 7. This finding supports those of Chessin et al. (1966) which showed peak RNA synthesis from 42-48 hours. Studies by Loeb et al. (1972), Rosenberg and Levy (1972), and Levy and Rosenberg (1973) demonstrated increases in protein synthesis shortly after mitogenic stimulation with continuation of protein synthesis through day 7. Since the scanning microscope photometry data showed changes in cell size and nucleic acid/protein content and heterogeneity, the finding that peak blast transformation occurred at day 5 suggested a combination of RNA, DNA and protein synthesis from 3-7 days with peak RNA synthesis possibly earlier than 3 days, decreasing through 7 days, peak DNA synthesis from 3-5 days and presumed maximal protein synthesis from 5-7 days.

In addition, lymphocytes from two subjects having severe combined immunodeficiency disease were also analyzed and compared to the normal individuals. One of the immunodeficient subjects, DS, displayed lymphocytes that were normal in size but packed with nucleic acid/protein, resulting in a higher than normal mean frequency.

The other immunodeficient subject, DV, showed a change in the structure and morphology of lymphocytes before and after treatment with transfer factor. Lymphocytes at six weeks of age were of two types: most were small cells with a "punched-out" chromatin network while some were larger, sparse "balloony" cells. Lymphocytes analyzed by SMP nine months after treatment with transfer factor gave a more normal appearance in terms of chromatin pattern as measured by nucleic acid/protein heterogeneity. The majority of the cells were larger and contained more nucleic acid/protein than normal, but in lesser proportions, resulting in a lower than normal mean frequency. This subject was also followed by electron microscopy which showed a more normal appearing population of cells four months after transfer factor treatment although 90% of the lymphocytes still contained membrane-bound immunoglobulins (B cells). Twelve months after transfer factor, 60% of the lymphocytes were positive for membrane-bound immunoglobulins and 3-7% were positive for the E rosette test (T cells). These findings indicated that transfer factor possibly aided in the maturation of the T lymphocyte population and/or caused structural changes in the B lymphocyte population

(Criswell et al., 1974). This change in cell types has been detected and followed by SMP over a two year period. The lymphocytes are becoming more normal in appearance even though clinical studies continue to give negative results. This subject will be followed over a period of time to monitor trends in cell types and provide quantitative data on nucleic acid/protein content and heterogeneity.

The two subjects diagnosed as having severe combined immunodeficiency disease presented two different morphologic pictures indistinguishable by functional tests. One subject showed lymphocytes normal in size but densely filled with nucleic acid/protein in a very heterogeneous distribution while the other subject showed lymphocytes larger and less dense than normal. These findings suggested the existence of at least two forms of immunodeficiency diseases, the pathologic defects of which may be very different. It has been postulated that immunodeficiency disease may be due to a buildup in nucleic acid/protein. This may occur due to a defect in the enzyme adenosin deaminase (Pluss et al., personal communication) or possibly to an imbalance in the DNA-histone fraction which causes stimulation of RNA synthesis (Mukherjee, 1970). The data has indicated that scanning microscope photometry may aid in the understanding of the basic defects involved, in differentiating various forms of immunodeficiency disease and in monitoring the treatment of immunodeficient individuals.

SUMMARY

In addition to immunologic, radioisotope incorporation and electron microscopic procedures, all research tools, the technique of scanning microscope photometry was shown to provide valuable information on individual lymphocytes, not discernable by the naked eye or available by any other means, to aid in the assessment of normal and abnormal lymphocyte morphology as well as assessment of immune responsiveness to mitogenic stimulation.

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III. Completed work on sectional phases (continued).

B. Virus Antigen Laboratory Findings:

Work on this project was performed in the Viral Diagnostic Laboratory at Methodist Hospital. The laboratory receives specimens from patients at Methodist Hospital, from the Harris County hospitals, Texas Children's Hospital, the M. D. Anderson Hospital, and from other institutions in the area. In collaboration with colleagues in pediatrics, research is ongoing in congenital cytomegalovirus infection. In addition to material from service patients, volunteers infected with influenza, adenoviruses, M. pneumoniae, rhinoviruses, and some other agents are intermittently available to provide material for evaluation of diagnostic methods.

Five cell lines are normally used for service diagnosis of viral infections: rhesus monkey kidney, human embryo kidney, rabbit kidney, human embryo fibroblasts (WI 38) and Hep-2 or HeLa. Results with cell culture provide the basis for comparison with other methods of diagnosis. M. pneumoniae may be isolated in cell cultures or on an artificial medium.

Detection of Antigen by Immunofluorescence.

Viral Infections of the Eye. Conjunctivitis is the most common disease of the eye. Its differential diagnosis includes bacterial infection, topical drug reaction, hypersensitivity involving the eye, and frequently adenovirus or herpesvirus infections. Since the management of these diseases is different and untoward effects can result if the wrong treatment is given, there is a need for early accurate diagnosis. For example, corticosteroids may be used in patients with adenoviral conjunctivitis, but this would be inappropriate for herpesvirus or bacterial infections of the eye.

Fourteen cases of acute conjunctivitis have been studied. In seven, diagnoses of viral infection (5 adenovirus, 2 herpesvirus) were made within a few hours by immunofluorescent detection of viral antigen from conjunctival exudate. The diagnoses were confirmed several days later by isolation of the agent in tissue culture. In four cases, no virus was identified by immunofluorescence antibody technique (IFAT) or in tissue culture. In three cases, adenoviruses were isolated after 8-10 days incubation in tissue culture.

Table 1

Diagnosis of Viral Conjunctivitis by Immunofluorescent Antibody Technique
Data on Positive Cases

Case No.	Name of Patient	Age, race, sex	Date onset initial symptoms	Results of viral diagnostic tests: Date obtained	IFAT[1]	Culture[2]
1	AR	64 W M	7-19-73	7-23-73	ADV	ADV
2	DK	29 W M	8-4-73	8-7-73	ADV	ADV
3	MF	21 W F	8-27-73	8-30-73	HSV	HSV
4	DS	28 W M	9-6-73	9-7-73	HSV	HSV
5	VR	39 W F	8-30-73	9-11-73	ADV	ADV
6	BS	22 W M	12-30-73	1-3-74	ADV	*
7	AJ	29 W F	1-3-74	1-4-74	ADV	*

1. Immunofluorescence testing requires only 2-3 hours.
2. Results with culture of adenovirus required 10 days or longer.

* Final diagnosis pending.

A report of one of these cases to illustrate the value of early diagnosis by IFAT is described below:

Case No. 3 (MF): This 21-year old female was initially examined on 8-30-73 with a 3-day history of pain, redness, and fine papillary eruption in the left medial canthal region with minimal conjunctival injection. She had noted no discrete vesicular lesions. Examination on 8-30-73 revealed best corrected vision of 20/20 in both eyes. There was a tender left preauricular node, minimal erythema and edema of the left medial canthal region, and follicular formation of the left lower tarsal conjunctiva. The cornea was normal. The clinical impression was atypical herpes simplex conjunctivitis.

Comment: The preauricular lymphadenopathy, follicular conjunctival change, and serous discharge in this patient resembled adenoviral conjunctivitis. There was no history of or signs of skin changes diagnostic of herpes simplex infection. The IFAT established the diagnosis of herpes simplex and prevented the incorrect use of topical corticosteroids as an anti-inflammatory measure.

Respiratory Viral Infections

Influenza. Last year details of the detection of influenza viral antigens in exudates were described. In the volunteers used as the basis of the study, titers of virus in exudates were so low that detection in nasal washes directly was of limited value.

With the prospect of the occurrence this year of new type A and B viruses, antiserum to these agents was prepared and is available. With our increasing experience in the collection of specimens rich in exfoliated epithelial cells, we feel that rapid diagnosis of these infections by IFAT of exudates will be possible.

Respiratory Syncytial Virus (RSV). Using commercial antiserum, RSV antigen has been identified in exudates from throat and trachea of six infants with bronchopneumonia. These findings have been confirmed by tissue culture (Hep-2). In sixteen other sick infants, both IFAT and cell culture for RSV were negative.

Respiratory Infections with M. pneumoniae. The first experiments we made for the detection of M. pneumoniae were very promising. Using a stock pool of the agent, we were able to show that even a small amount of mycoplasma could be detected in three days in VERO cell culture:

Table 2

Immunofluorescence of Mycoplasma pneumoniae in Monkey Kidney Continuous Cell Culture (VERO)

Inoculum 0.2 ml	Day 1	Day 2	Day 3	Day 5	Day 7
$10^{6.3}$ CFU/ml	2+	2+ – 3+	4+		
$10^{4.3}$	1–2+	2+	3+	4+	
$10^{2.3}$	1+	1–2+	2+–3+	3+–4+	4+
$10^{1.3}$	0	±	1+	1+–2+	3+

Note: Inoculum from a stock pool was added in amounts described in column 1. One to four plus indicates the amount of specific fluorescence.

but using wild specimens we haven't been able to get results until seven days. Although this is earlier than detection by the regular method (2 weeks are usually necessary), it is not rapid enough to qualify as rapid diagnosis. We are now working on several factors that might improve the growth of the mycoplasma in tissue culture, such as type and concentration of serum in the medium, and length of absorption of the specimens onto the cells.

Herpesvirus. Fifteen cerebrospinal fluid specimens from patients with acute central nervous system disease were tested for herpesvirus antigen. Three of these were positive for HSV fluorescence of sedimented CSF. Cell cultures of the spinal fluids of these patients were negative (although the agent was isolated from lesions in the brain or elsewhere in the body. One of these cases represented disseminated disease from a penile lesion with Type II HSV. A summary of his case follows:

D.T. 24 year old WM

Presented with 4-month history of painful vesicular penile lesions. One week prior to admission also noted the onset of vesicles on the left side of his forehead. He also had 3 days of frontal headache, malaise and fever to 101^0.

On physical examination, he had scattered vesicles on glans penis and forehead. He had no neck rigidity of Babinski sign. Cerebrospinal fluid revealed 270 WBC, all mononuclears. Protein and sugar were normal. All cultures were negative for TB, bacteria, fungi, and viruses. Immunofluorescence of white cells in CSF were positive for Herpes simplex virus. A virus culture of vesicle fluid grew Herpes hominis virus type II.

Headache and fever slowly regressed over 4 days. Photodynamic inactivation of the cutaneous herpetic lesions was carried out with proflavine.

Epstein-Barr (E.B.) virus antibody. Evidence now strongly supports the concept that infectious mononucleosis is caused by primary infection with a virus antigenically related to E.B. virus. The relation of this syndrome to asymptomatic carriage of the virus and to certain tumors (Burkitt's tumor especially has not been clarified. Nevertheless, the epidemiology of infection with E. B. virus is an important research topic and in some cases the test may specifically diagnose infectious mononucleosis.

We obtained a culture of E.B.-3 cells. This line produces coat proteins of E.B. virus in cell culture and was originally obtained from a specimen of Burkitt's tumor. Dilutions of test sera applied to E.B.-3 cells dried on a microscope slide readily fluoresced by the indirect method. Titers of 1:160 or greater are suggestive of recent infection, and paired specimens showing a 4-fold or greater rise in titer of antibody are diagnostic of infection with this virus. The significance of these tests in various syndromes of illness will continue under investigation.

Rubella. The rubella virus is difficult to isolate and requires at least fourteen days of incubation before it can be detected in tissue culture. We inoculated rubella in a continuous line of rabbit kidney cells (RK 13). After five days, specific immunofluorescence was detected with the use of commercially available rubella equine antiserum and fluorescent antihorse globulin. There is a signivicant amount of nonspecific fluorescence due to antibody to antigens of the cell cultures. Studies are under way to improve the sensitivity and specificity of this test.

Radioimmunoassay.

(This work was done in this laboratory by Dr. J. A. Kasel and was not supported by the NASA contract.)

Influenza virus antigen was deposited on the walls of plastic dishes with multiple small receptacles. The deposited virus was incubated with dilutions of known positive and negative sera and later treated with ^{131}I tagged antihuman globulin. Antigen could be easily detected in this system through a wide range of dilutions. In the coming year we will attempt to use this methodology in detection of viral antigens from several different infections.

Summary and Recommendations for Future Research.

During the period of the contract, we have established procedures for immunofluorescent identification of nine agents in exudates or cell cultures inoculated with exudates from patients: Influenza type A and B, parainfluenza I, II, and III, respiratory syncytial virus, herpesviruses, adenoviruses, and M. pneumoniae. There is, in general, high specificity and sensitivity of these tests. It is necessary, however, to gain more experience and to attempt to improve the methods used with some of the agents, especially rubella and M. pneumoniae. Further work is also planned of IFAT with cytomegalovirus.

Detailed comparisons of the IFAT with conventional tissue culture methods should be made. We believe IFAT will make it possible to reduce the use of cell culture and reduce the overall cost of virus diagnosis. It is believed this can be done at the same time with improved speed of diagnosis and nearly equivalent sensitivity.

The use of radioimmune assay should be explored by making quantitative comparisons of this mode of diagnosis with IFAT and cell culture.

It may be possible also, with the use of viral infections induced in volunteers, to compare the gas chromatographic abnormalities detectable by Zlatkis in patients' serum and urine with alterations specifically due to infection.

The coronavirus, the most recently discovered respiratory viral infection, is responsible for a considerable number of cases of respiratory illness, especially in older children and young adults. The detection of this agent has been hindered by its fastidious growth requirements. In the coming year, special effort should be made to isolate some of these viruses, and attempt should be made to improve their methods of detection.

III. Completed work on sectional phases (continued).

C. Macrophage Findings:

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I. Pulmonary Macrophages

A. General Observations:

We have continued to study pulmonary alveolar macrophages (PAMs) obtained by pulmonary lavage through an Olympus Fiberoptic bronchoscope. A total of 45 nonsmokers and 24 cigarette smokers have now been studied, and the yield of cells obtained by lavage is very similar to that reported in the previous annual report of this contract (Table I). We continue to note a higher yield of cells from the smokers than from the nonsmokers. The percentage of PAMs obtained from cigarette smokers was higher than from nonsmokers, while a higher percentage of lymphocytes were obtained from nonsmokers than from smokers. The percentage of small mononuclear cells (resembling blood monocytes) was similar in both groups. When the total number of cells obtained is calculated, the total yield of lymphocytes was almost twice as high in smokers and the small mononuclear cells were increased four-fold in the lavage from smokers (Table II).

In the course of these investigations, several of the volunteers were lavaged on multiple occasions (3 to 7 times). All of these individuals were nonsmokers. As might be expected, there was variability in the yield of cells recovered from the lavage (Table III). The variation in the amount of lavage fluid recovered was reasonably small (about 6.8%), but the variation in cell yield ranged from 23.5 to 50.4% (Table IV).

Since varied morphology is observed in macrophages obtained from healthy volunteers (and in order to evaluate further the effects of smoking on these cells), the PAMs from 12 nonsmokers and 11 cigarette smokers were classified using the morphological criteria described in the previous annual report.(Fig.1) Briefly restated, the morphological characteristics are as follows: Type 1 cells have little or no cytoplasmic inclusions, while Type 2 cells have small but discrete inclusions, with no brownish discoloration of the cytoplasm. Type 3 cells have small or moderate size inclusions, but they also have a definite brown sheen throughout the cytoplasm of the cell. Type 4 cells have cytoplasmic inclusions of varying size as well as a dark brownish sheen. The percentage of the PAMs belonging to each of these morphological types is presented in Figure 2. While nonsmokers and smokers had each of these types of cells represented in the lavage, the Type 1 cells accounted for 74% of the PAMs from nonsmokers while only 6% of PAMs from cigarette smokers were Type 1 cells. There was also a marked difference in the percentage of Type 4 cells. These cells with large amounts of inclusion material made up 64% of the smokers PAMs, but only 2% of the nonsmokers PAMs. When these percentages are used to calculate the numbers of each type of cell obtained from nonsmokers and from

TABLE I. Cells Obtained by Pulmonary Lavage

	No. free pulmonary cells/cc lavage recovered*	% Pulmonary alveolar macrophage	% Lymphocytes	% Small Mononuclear cells
Nonsmokers (N = 45)	$15.0 \pm 9 \times 10^4$	81.7 ± 1.5	12.8 ± 1.4	4.0 ± 0.4
Smokers (N = 24)	$55.5 \pm 5.6 \times 10^4$	87.0 ± 1.5	6.5 ± 1.1	3.7 ± 0.5

TABLE II. CELLS RECOVERED BY 250 ml BRONCHIAL LAVAGE*

	Total free Bronchial Cells	Total PAM	Total Lymphocytes	Total Small Mononuclear
Nonsmokers (N = 45)	$21.8 \pm 1.6 \times 10^6$	$17.8 \pm 1.3 \times 10^6$	$28.9 \pm 4.2 \times 10^5$	$8.2 \pm 0.9 \times 10^5$
Smokers (N = 24)	$81.7 \pm 7.8 \times 10^6$	$71.5 \pm 7.1 \times 10^6$	$48.3 \pm 8.8 \times 10^5$	$30.1 \pm 6.6 \times 10^5$
P value	<.001	<.001	<.05	<.001

* Mean recovery of 250 ml saline lavage: 160 ± 5 ml (both groups)
Students t test for non-paired data (cells/cc lavage recovered:
$t = 9.42733$, $P = < .001$

TABLE III

INDIVIDUAL VARIATIONS WITH REPEATED ENDOBRONCHIAL LAVAGE

Nonsmokers	Lavage Vol. Recovered	%Return	# Cells/ml	Total # Cells	%PAM	%Lymph	%s-MN	Total # Recovered PAM	Lymph	s-MN
P. W. std. error N = 7	181.4 ml ± 9.2 ml	72.6 ± 5	1.34×10^5 $\pm 2.8 \times 10^4$	2.55×10^7 $\pm 5.7 \times 10^6$	84.1 ± 1.1	10.6 ± 1.2	4.4 ± 1.0	2.2×10^7 $\pm 5.0 \times 10^6$	2.5×10^6 $\pm 6.4 \times 10^5$	1.1×10^6 $\pm 3.8 \times 10^5$
F. K. std. error N = 3	165 ml ± 12.7	66 ± 5.1	1.87×10^5 $\pm 8.0 \times 10^3$	3.13×10^7 $\pm 3.5 \times 10^6$	83.3 ± 7.8	14.0 ± 8.1	2.7 ± 1.5	2.6×10^7 $\pm 1.2 \times 10^6$	4.7×10^6 $\pm 3.2 \times 10^6$	7.9×10^5 $\pm 4.1 \times 10^5$
E. C. std. error N = 4	145 ml ± 10.3	58 ± 4.1	1.60×10^5 $\pm 4.7 \times 10^4$	2.60×10^7 $\pm 9.5 \times 10^6$	79.8 ± 2.6	16.2 ± 3.4	3.2 ± 0.7	2.0×10^7 $\pm 7.0 \times 10^6$	4.9×10^6 $\pm 2.3 \times 10^6$	7.5×10^5 $\pm 2.4 \times 10^5$
K. F. std. error N = 5	159.0 ml ± 16.6	63.6 ± 6.6	1.70×10^5 $\pm 4.5 \times 10^4$	2.46×10^7 $\pm 4.5 \times 10^6$	79.8 ± 5.4	12.8 ± 5.6	6.0 ± 0.8	2.0×10^7 $\pm 4.0 \times 10^6$	3.0×10^6 $\pm 1.6 \times 10^6$	1.4×10^6 $\pm 2.5 \times 10^5$
M. C. std. error N = 4	145 ml ± 5.8	58.0 ± 2.3	5.95×10^4 $\pm 2.0 \times 10^4$	8.67×10^6 $\pm 3.0 \times 10^6$	87.2 ± 4.0	9.0 ± 3.7	3.2 ± 1.2	7.8×10^6 $\pm 2.7 \times 10^6$	7.4×10^5 $\pm 4.3 \times 10^5$	2.7×10^5 $\pm 1.5 \times 10^5$

* N = Number of individual times a subject was lavaged; with a minimum of two months separating successive lavages.

TABLE IV

INDIVIDUAL VARIATIONS WITH REPEATED ENDOBRONCHIAL LAVAGE

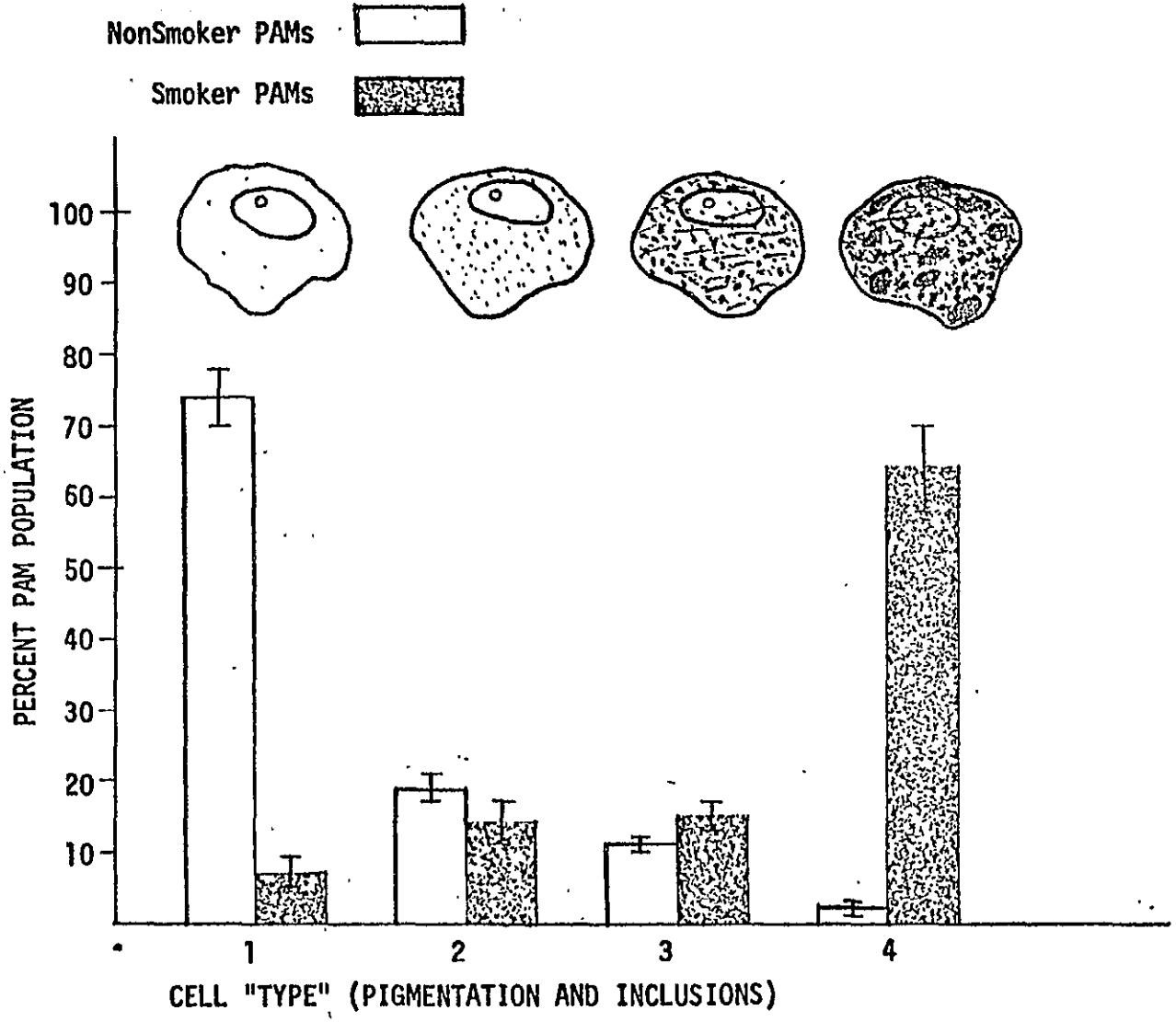
	Lavage Volume Recovered	No. free pulmonary cells/cc lavage recovered	Total free bronchial cells
D:	6.8 ± 1.2%	22.9 ± 5.7%	24.3 ± 5.3%
	Total PAM	**Total lymphocytes**	**Total small mononuclear**
D:	23.5 ± 6.2%	50.4 ± 7.9%	38 ± 7.8%

Variation of values as percent of means obtained for five nonsmokers lavaged on at least three separate occasions.

$$D = \left[\frac{\Sigma\ (\text{std. error}_n \div \text{mean}_n)}{5}\right] \times 100$$

FIGURE 1

Morphologic Variation in PAMs
Lavaged from a Cigarette Smoker



Distribution of pulmonary alveolar macrophages

FIGURE 2

smokers (taking into account the marked increase in the number of cells recovered from the smokers) there is still a three-fold greater number of Type 1 cells from nonsmokers than from smokers. The number of cells in each of the other morphological categories was much higher in the material lavaged from smokers than from nonsmokers (Table V).

When histochemical staining for acid phosphatase was performed, there was a marked increase in reactivity of the PAMs from cigarette smokers compared to the acid phosphatase reaction in PAMs from nonsmokers (Figure 3). This is consistent with the early observation that acid hydrolase activity is regularly increased in PAMs from cigarette smokers. When the percentages are used to calculate the numbers of acid phosphatase reactive cells obtained from each group, the increase in smokers cells is even more marked (Table VI). Although not specifically tested in these studies, it is likely that other acid hydrolase activities are elevated to a comparable degree in PAMs from smokers.

Finally, when histochemical staining for lipid (using Sudan Black B) was performed, the reaction product was much more intense in PAMs from smokers than from nonsmokers (Figure 4).

The inclusion material in the PAMs of cigarette smokers is auto-fluorescent when viewed under the fluorescent microscope (Figure 5). In this property, as well as the histochemical staining characteristics, the inclusion material resembles lipofuscin.

1. Surface and Membrane Characteristics of PAMs: Preliminary observations have been made on the effects of cigarette smoking on the surface morphology of PAMs from normal volunteers. A cluster of macrophages from a nonsmoker is illustrated in Figure 6, while the scanning electron micrograph of characteristic PAMs from cigarette smokers appears in Figure 7. The PAMs from smokers regularly have smoother surfaces with fewer membrane projections than comparable cells from nonsmokers. These morphological differences may have certain functional consequences. The number of receptor sites on the membrane may be reduced in the smoother cells from cigarette smokers. In particular, the number of receptor sites for complement seem to be diminished in PAMs from cigarette smokers, as illustrated in Figure 8. In evaluating receptor sites, the formation of red blood cell rosettes was quantitated after preincubation with complement and antigen. While a smaller percentage of cells from smokers have receptor sites for the EAC complex, when the appropriate adjustments are made, the number of rosette forming cells is similar in both groups of normal subjects (Figure 8).

TABLE V

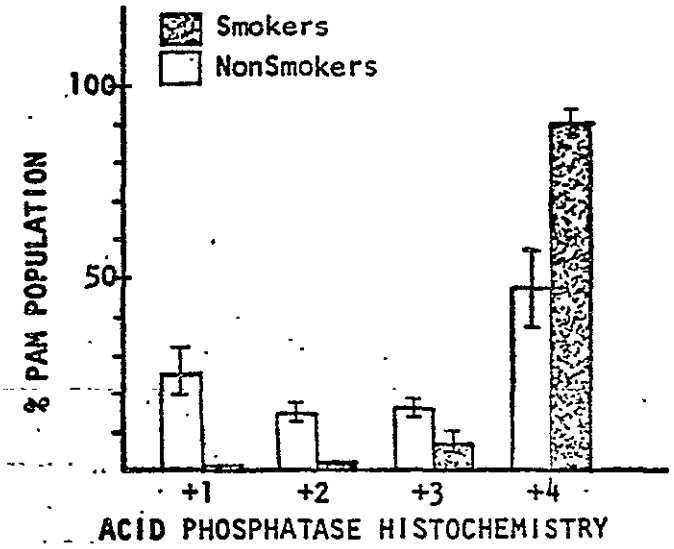
DISTRIBUTION OF PULMONARY ALVEOLAR MACROPHAGES

Quantitation of Cell "Type" (Pigmentation and Inclusions)*

	Type 1	Type 2	Type 3	Type 4
NonSmokers	1317.2 ± 52.7 x 10^4	320.5 ± 6.4 x 10^4	195.8 ± 2.0 x 10^4	35.6 ± 0.4 x 10^4
Smokers	429 ± 8.6 x 10^4	1001.0 ± 30.0 x10^4	1501.5 ± 30.0 x 10^4	4576.0 ± 228.8 x 10^4
Ratio Nonsmoker:Smoker cells	3.1:1	1:3.1	1:7.7	1:128.5

*Total numbers recovered by lavage with 250 ml saline, with a mean recovery volume of 160 ± 5 ml.

FIGURE 3



Distribution on acid phosphatase reactivity in PAMs from NonSmokers and Smokers

TABLE VI

QUANTITATION OF ACID PHOSPHATASE HISTOCHEMISTRY

	Acid Phosphatase Reactivity of PAMs*			
	+1	+2	+3	+4
NonSmokers	$4.45\pm0.20\times10^6$	$2.67\pm0.05\times10^6$	$2.85\pm0.57\times10^6$	$8.37\pm0.67\times10^6$
Smokers	$7.15\pm0.001\times10^5$	$1.43\pm0.07\times10^6$	$5.01\pm0.10\times10^6$	$6.44\pm0.19\times10^7$
Ratio NonSmokers:Smokers	6.2 : 1	1.8 : 1	1 : 1.7	1 : 7.6

* Total numbers recovered by endobronchial lavage with 250 ml Normal Saline, with a mean recovery volume of 155 ± 5 ml

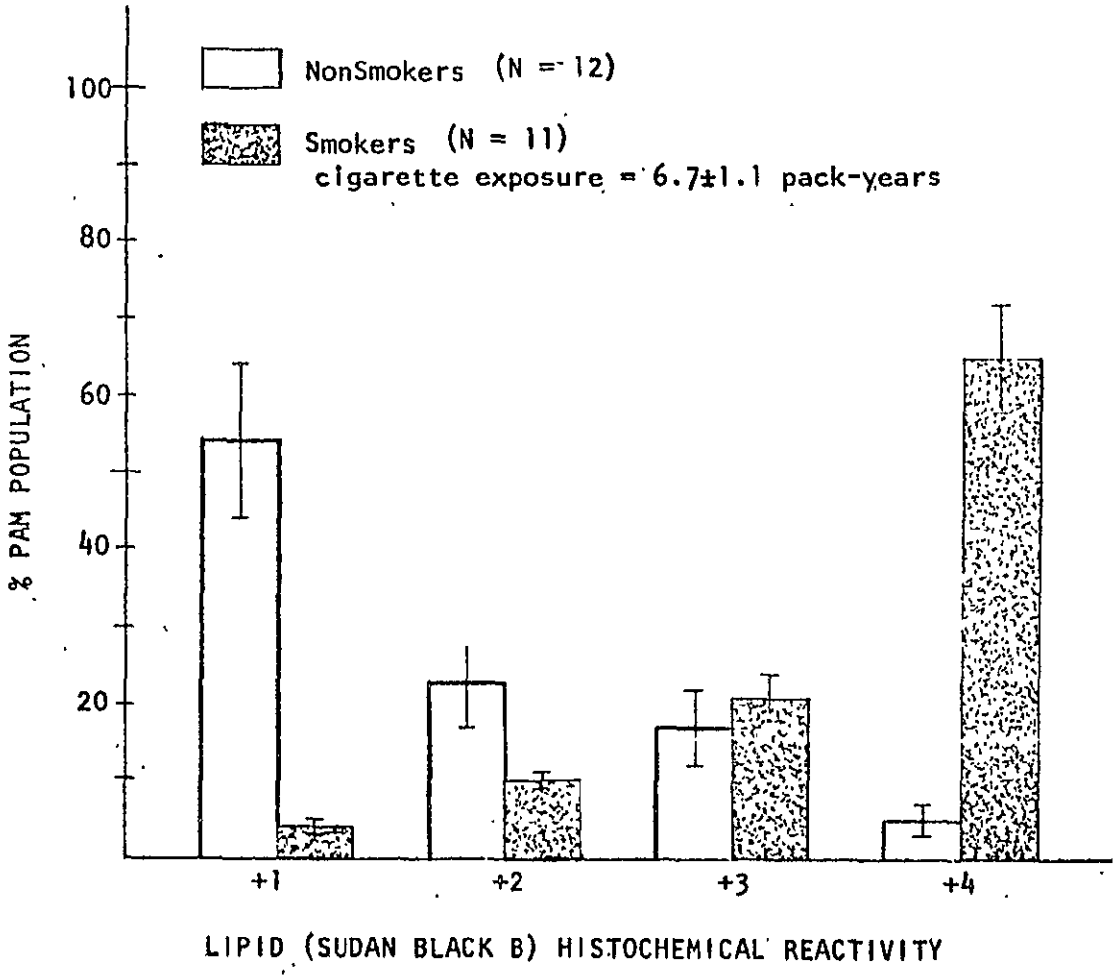

NonSmokers (N = 12)
Smokers (N = 11)
cigarette exposure = 6.7±1.1 pack-years
100
80
60
40
20
% PAM POPULATION
+1
+2
+3
+4
LIPID (SUDAN BLACK B) HISTOCHEMICAL REACTIVITY

FIGURE 4

FIGURE 5

Autofluorescent Granules in PAMs
From Cigarette Smokers

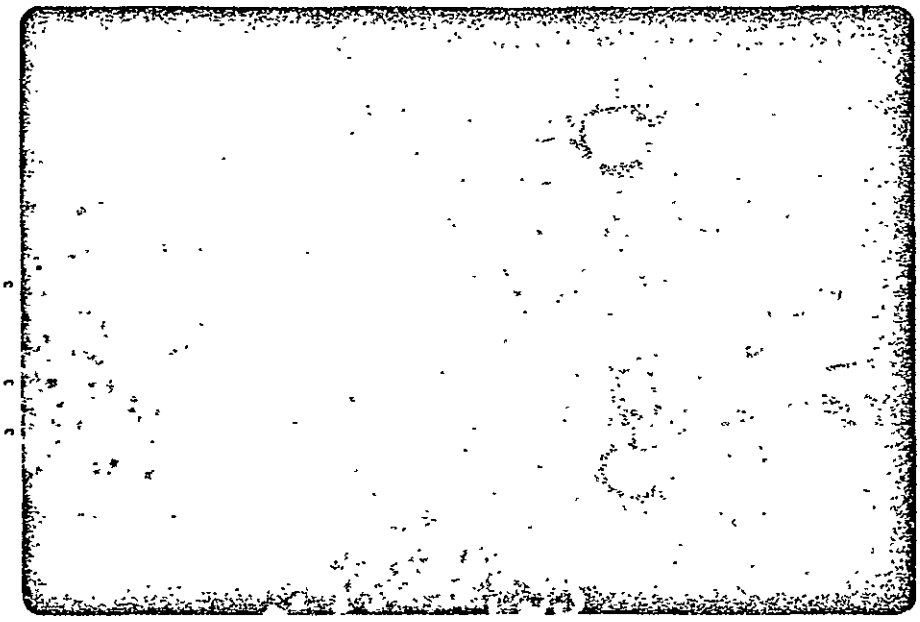


Figure 6

Human Pulmonary Alveolar Macrophages from a Nonsmoker

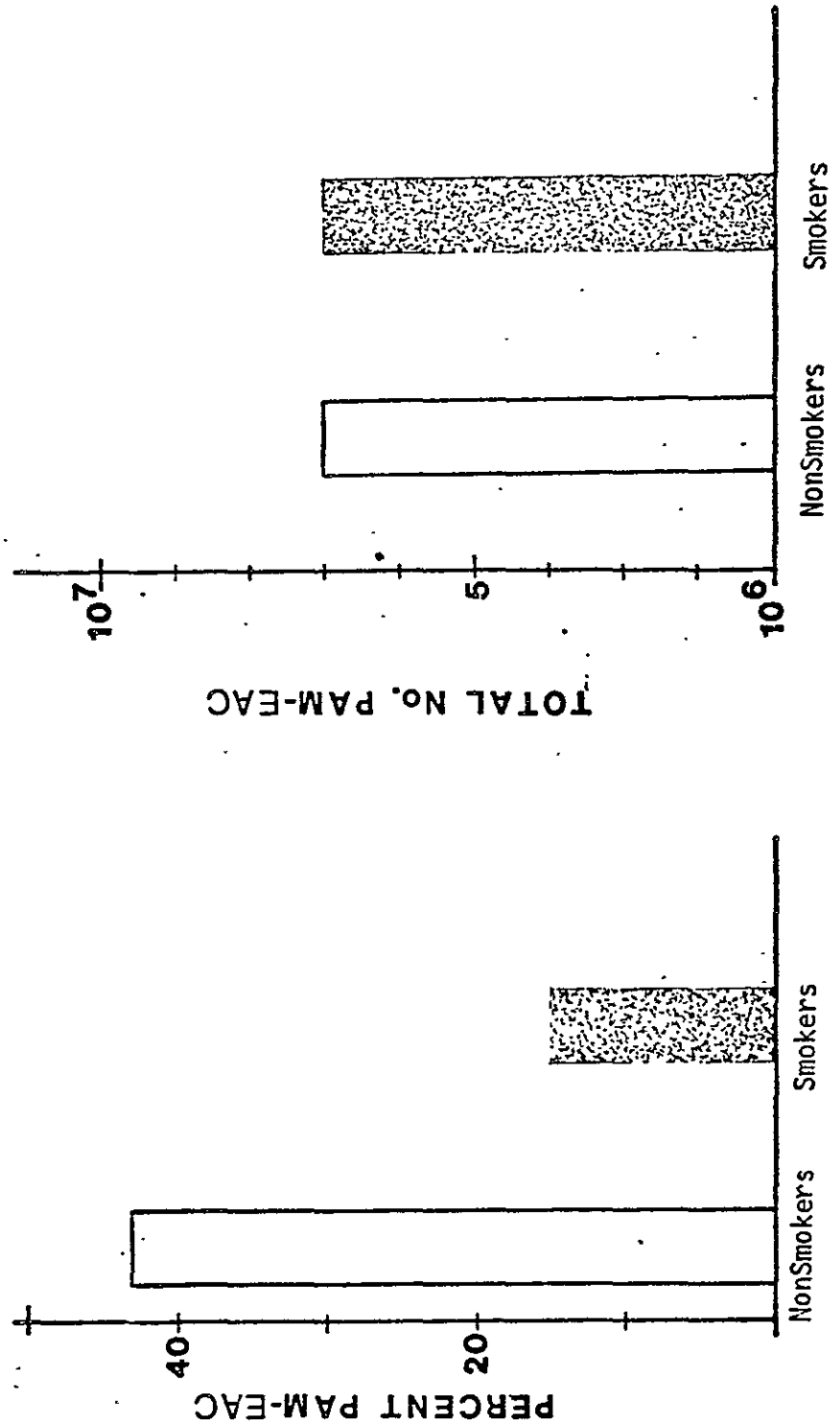
Scanning Electron Photomicrograph (5100 x Mag.)

Figure 7

Human Pulmonary Alveolar Macrophages from a Cigarette Smoker

Scanning Electron Micrograph (4300 x Mag.)

FIGURE 8


TOTAL No. PAM-EAC
10^7
5
10^6
Smokers
NonSmokers
PERCENT PAM-EAC
40
20
Smokers
NonSmokers

2. Bronchial Lymphocytes: In addition to the PAMs, the cell type most intensively studied, bronchial lavage yields a considerable number of lymphocytes. Since there is growing evidence that interactions between lymphocytes and macrophages within the lung may be important in a variety of pathological conditions, we have begun to study the bronchial lymphocytes and to try to characterize some of the effects of cigarette smoking on these cells.

In recent years two major subgroups of lymphocytes have been identified; the lymphocytes responsible for cell mediated immunity which develop under the influence of the thymus, and are called T cells and those lymphocytes which are responsible for antibody production and are influenced by the equivalent of the bursa, and are termed B cells. We obtained peripheral blood and bronchial lavage specimens from a group of nonsmokers and cigarette smokers to determine the relative distribution of both types of lymphocytes in these two different tissues in the same individual. The T cells were identified by their ability to form rosettes when incubated with sheep erythrocytes. B lymphocytes were identified by the formation of rosettes with sheep erythrocytes coated with antibody and complement. Some studies were also performed in which surface immunoglobulin was identified on lymphocyte surfaces by fluorescent microscopy. Lymphocytes which did not show any of these reactions were termed null lymphocytes. The distribution of lymphocytes in the peripheral blood of the smokers and nonsmokers was similar (Table VII). About half of the lymphocytes in the peripheral blood of both subjects were T cells, while B cells accounted for about 15% of the lymphocyte population and about 1/3 of the lymphocytes were null cells. The bronchial lymphocytes obtained from nonsmokers were significantly different in their distribution than the peripheral blood lymphocytes from the same individuals, demonstrating a reduction in both T cells and B cells (with a corresponding increase in the null cell population. In contrast, the bronchial lymphocytes from cigarette smokers had a higher percentage of T cells (approaching that observed in the peripheral blood). Since the number of lymphocytes obtained from cigarette smokers is higher than the number obtained from nonsmokers, when the percentages are used to calculate the total number of lymphocytes recovered from each group, the number of B cells and null cells from smokers and nonsmokers are almost identical, while the increase in T cells accounts almost entirely for the increased number of lymphocytes recovered from cigarette smokers (Figure 9).

An alternative method of classifying lymphocytes is based on the surface morphology by scanning electron microscopy. The surface of B lymphocytes has numerous projections, while the surface of T lymphocytes is relatively smooth with fewer membrane projections. An example of surface morphology of a sample of bronchial lymphocytes is presented in Figure 10. In a small number of studies of the surface morphology of bronchial lymphocytes, the percentage of T cells was

increased in the lavage obtained from cigarette smokers (Table VIII). The criteria for separating cells into either B cells or T cells using scanning electron microscopy is much more subjective than the previous method, and these problems may account for differences in the absolute percentages obtained by this technique when compared with other techniques for classifying lymphocytes.

While more work will be required before the significance of these alterations in lymphocytes can be understood, it is probable that the shifts in lymphocyte populations are a reflection of irritation and inflammation which accompany smoking.

B. Functional Studies:

In addition to the previously described morphological and biochemical studies, attempts have been made to evaluate the in vitro responsiveness of PAMs using several different techniques to measure function. Since it is important to know the range of variation in normal individuals, and to evaluate the possible effects of cigarette smoking on function, we have continued our comparisons between cells obtained from nonsmokers and from cigarette smokers. Two major techniques have been employed. Migration of PAMs, and responsiveness of these cells to a chemotactic stimulus has been measured in a modification of the Boyden micropore filter chamber (using methods described in the previous annual report). The phagocytosis of the staphylococci by PAMs has been evaluated microscopically, using an autoradiographic technique.

1. Migration and chemotactic responsiveness: The migration of PAMs was measured using a double membrane chamber, with incubations of 18 hours at 37° . When incubated in a similar fashion and for the same time period, over twice as many macrophages from cigarette smokers as from nonsmokers migrated through the 14 micron pore size nylon filter employed in the chambers (Figure 11). When casein was present as a chemotactic stimulus in the lower compartment of the chamber, the increase in migration of smokers PAMs was more marked than the increase in migration of PAMs from nonsmokers (Figure 12). These observations tend to confirm the prior studies using other techniques which indicated that PAMs from cigarette smokers are more active cells with higher metabolic requirements and more active in vitro migration. The responsiveness to casein indicates that these cells can continue to be influenced by the environment, and that they are capable of directing their migration in response to external chemotactic stimuli.

2. Phagocytosis of staphylococci: One of the most widely accepted roles for the PAM involves protection of the host by ingestion of foreign material (including microorganisms) which enter the lung through the respiratory tract. Since the morphology of PAMs is markedly altered by cigarette smoking, it is important to determine if smoking also influences the phagocytic abilities of these cells.

TABLE VII

LYMPHOCYTES IN NON-SMOKERS

AND CIGARETTE SMOKERS

	Non-Smokers		Smokers	
	Blood	Lung	Blood	Lung
	%	%	%	%
T-Lymphocytes (E)	50.3	17.6	48.5	43.0
B-Lymphocytes (EAC)	15.3	3.5	15.2	5.3
B-Lymphocytes (FA)	16.0	2.0	16.0	N.D.
N-Lymphocytes (null)	34.0	78.9	36.0	49.0

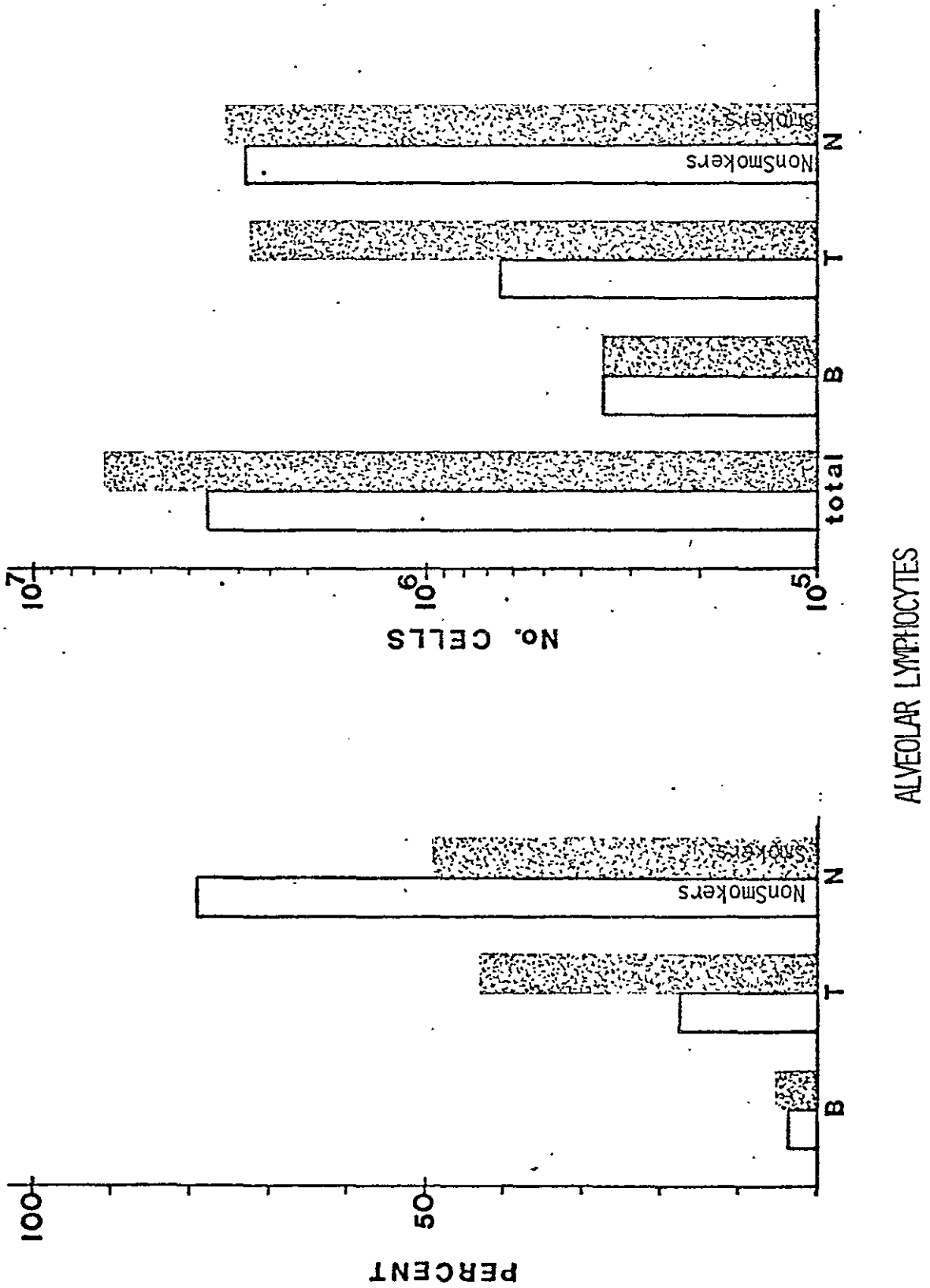
TABLE VIII

PULMONARY ALVEOLAR LYMPHOCYTE CLASSIFICATION

BY SCANNING ELECTRON MICROSCOPY

	Percent Lymphocyte Population	
	B-cells	T-cells
Nonsmoker	41%	49%
Smoker	24%	76%

FIGURE 9


ALVEOLAR LYMPHOCYTES
No. CELLS
10^7
10^6
10^5
total
B
T
N
Smokers
NonSmokers
PERCENT
100
50
B
T
N
Smokers
NonSmokers

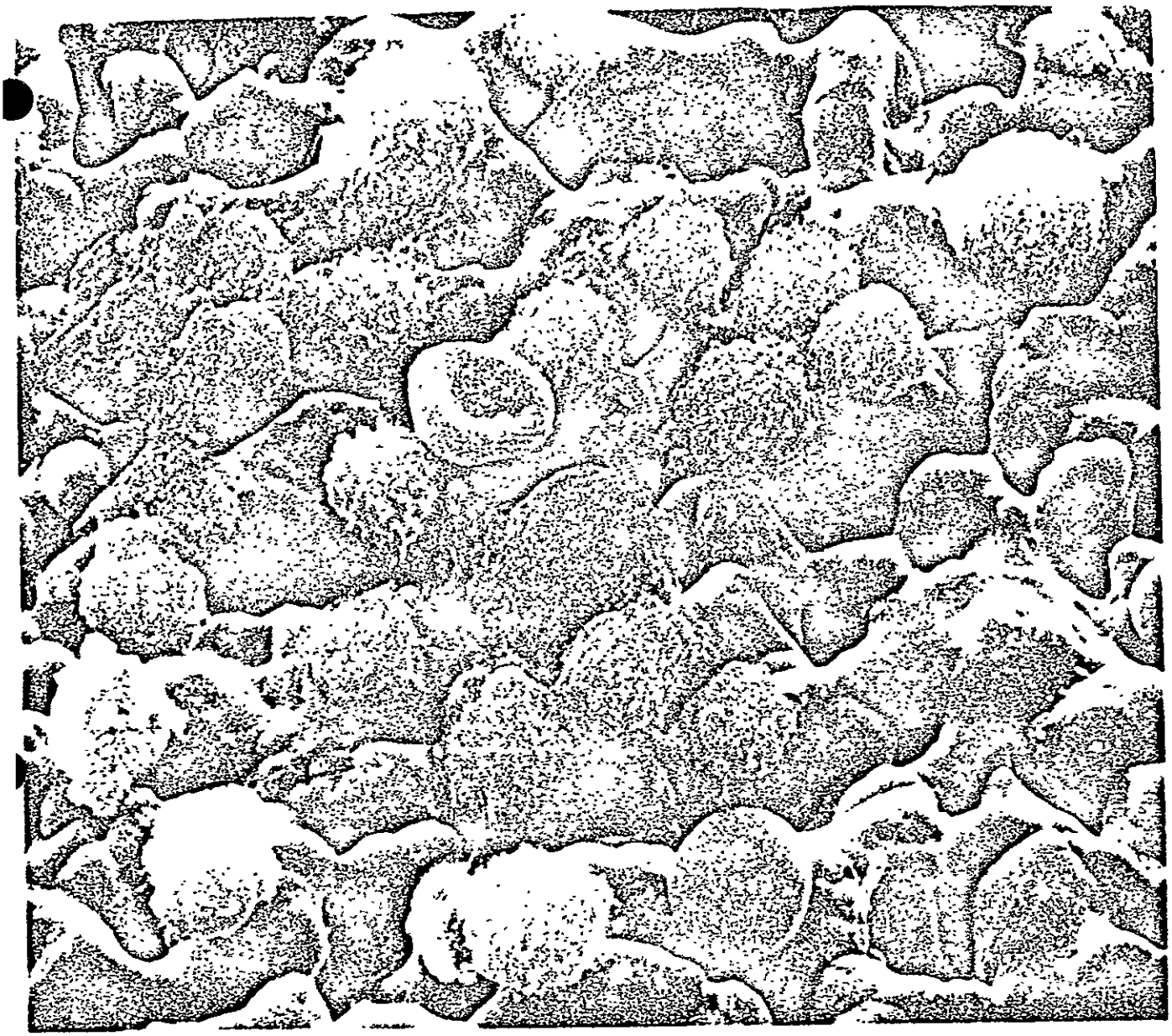


Figure 10

Human Pulmonary Alveolar Lymphocytes

Scanning Electron Photomicrograph (4100 x Mag.)

FIGURE 11

Migration of PAMs in vitro

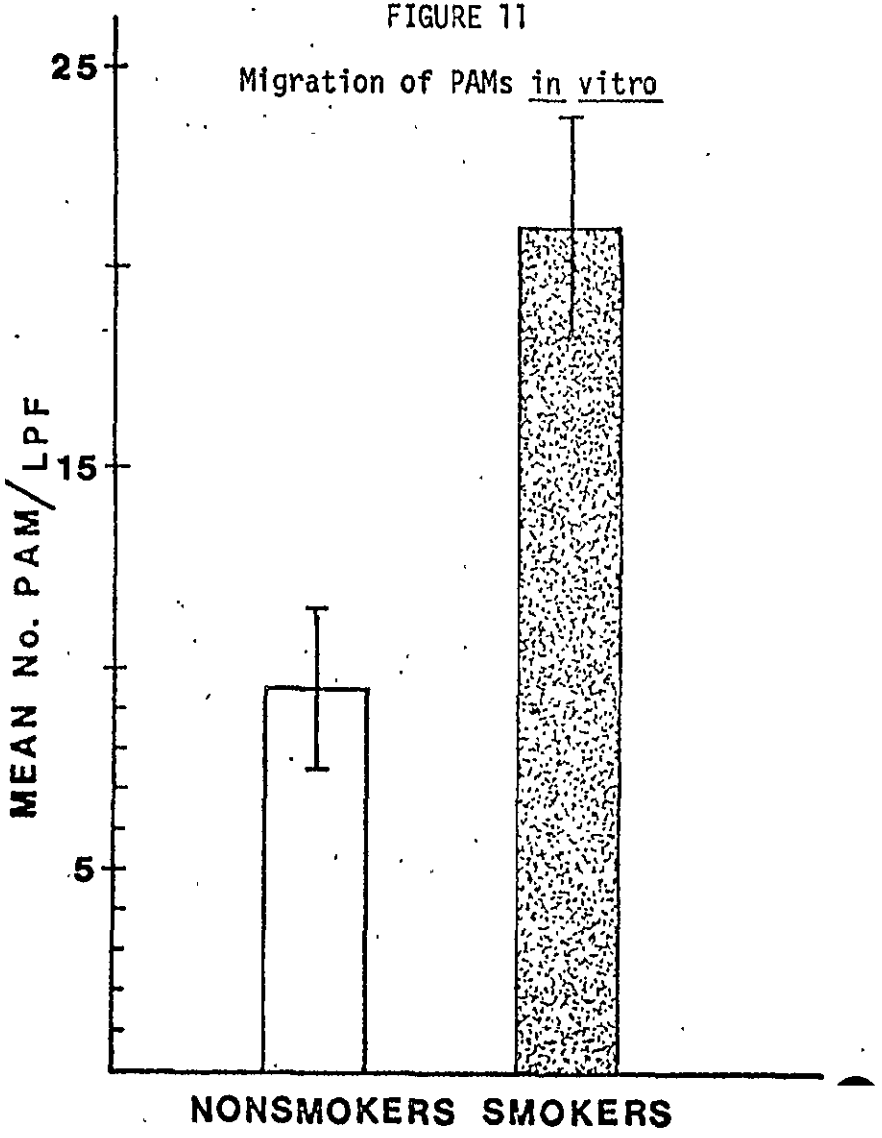
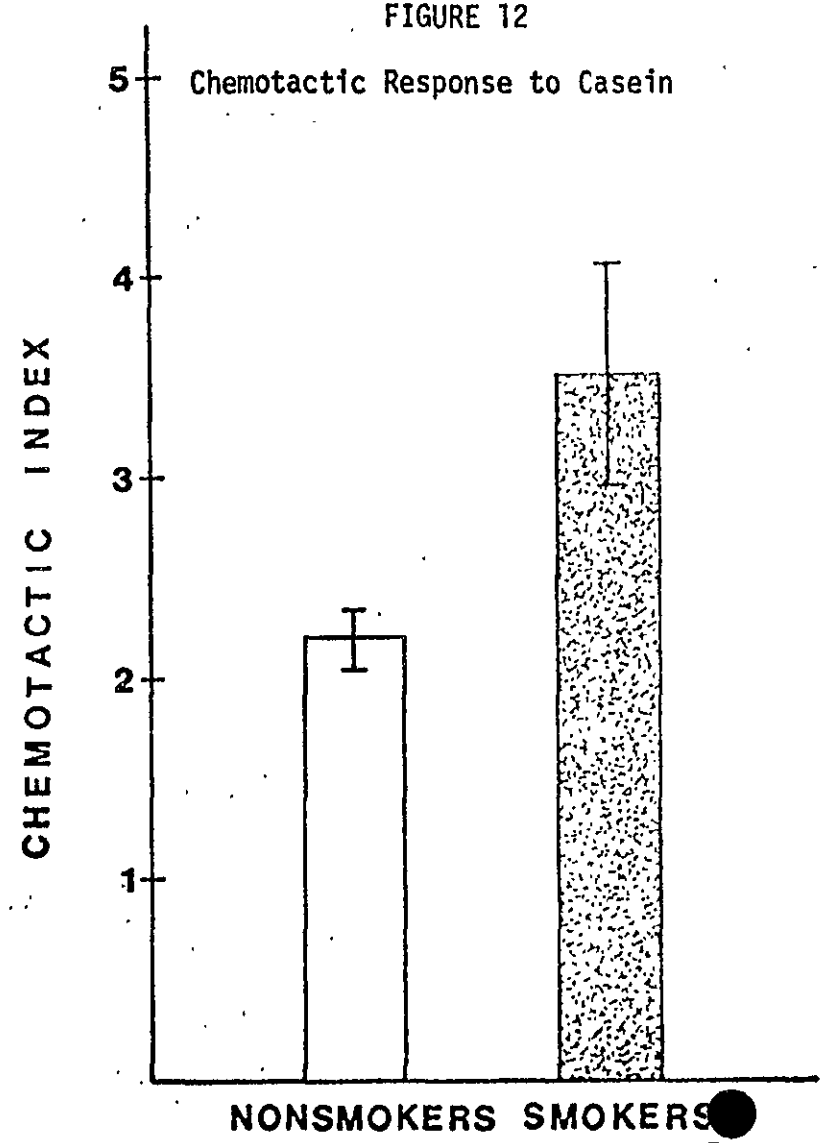


FIGURE 12

Chemotactic Response to Casein



The technique chosen to evaluate phagocytosis of macrophages is an adaptation of the procedure outlined in the annual report submitted in August 1973. A flow sheet summarizing the steps in this procedure is illustrated in Figure 13. Briefly described, bacteria in the log phase of growth are added to a monolayer of macrophages which have been preincubated and attached to glass in a tissue culture chamber slide. Phagocytosis is allowed to progress for varying lengths of time, then the extracellular bacteria are washed off the cells and tritiated thymidine is added. Slides are incubated at 37° for an additional 20 minutes, after which free thymidine is removed by washing six times. The plastic chamber is removed and the slides are processed for autoradiography and staining. Particle uptake is quantitated microscopically, and the percentage of intracellular bacteria which have incorporated thymidine is determined. A bacterial control is run simultaneously using the initial log phase culture to determine the uptake of thymidine by the extracellular bacteria. The reduction in thymidine incorporation by bacteria that have been ingested by the phagocytic cells is then calculated.

Studies were performed using PAMs obtained from nonsmokers and from cigarette smokers to determine the possible effects of cigarette smoking on phagocytosis of staphylococci. These cells were incubated in medium containing 10% autologous serum, heated serum, or in medium containing no human serum. In all three incubation conditions, macrophages from cigarette smokers demonstrated less phagocytosis of staphylococci than PAMs from nonsmokers (Figure 14). In addition to having a lower percentage of cells which were phagocytic, the individual PAMs from smokers took up fewer bacteria per cell than the PAMs from nonsmokers (Figure 15). Again, the results were similar whether the system contained normal autologous serum, heated serum, or no added serum. Over half of the phagocytic PAMs from cigarette smokers contained four or fewer bacteria, while the majority of PAMs from cigarette smokers contained five or more bacterial particles. Therefore, cigarette smoking is associated with a reduction in the phagocytosis of staphylococci, which may be related to the previously demonstrated morphological changes (fewer surface projections, and larger number of cytoplasmic inclusions).

Since differences have been described in the phagocytic abilities of mononuclear cells in the peripheral blood (with monocytes from females being consistently more active as phagocytes), we reviewed our phagocytosis data to see if the sex of the donor had any influence on the results observed. There was no significant difference in phagocytic abilities of PAMs obtained from male volunteers or from female volunteers (Figure 16). Similarly, the number of bacteria ingested by each phagocytic cell was comparable whether the PAMs were from a male or a female (Figure 17). In the system employed, there is no demonstrable difference in phagocytic function of PAMs from males and females.

FIGURE 13

PHAGOCYTOSIS PROCEDURE

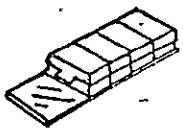
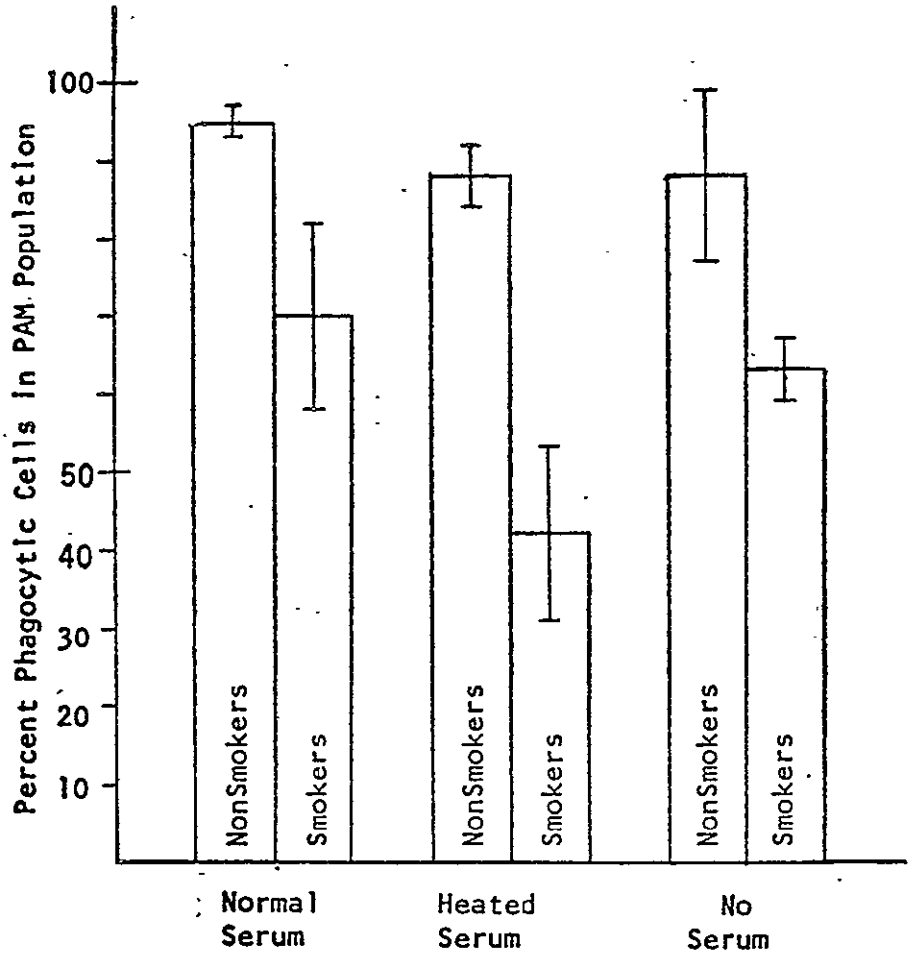


FIGURE 14


Percent Phagocytic Cells In PAM Population
100
50
40
30
20
10
NonSmokers
Smokers
NonSmokers
Smokers
NonSmokers
Smokers
Normal Serum
Heated Serum
No Serum

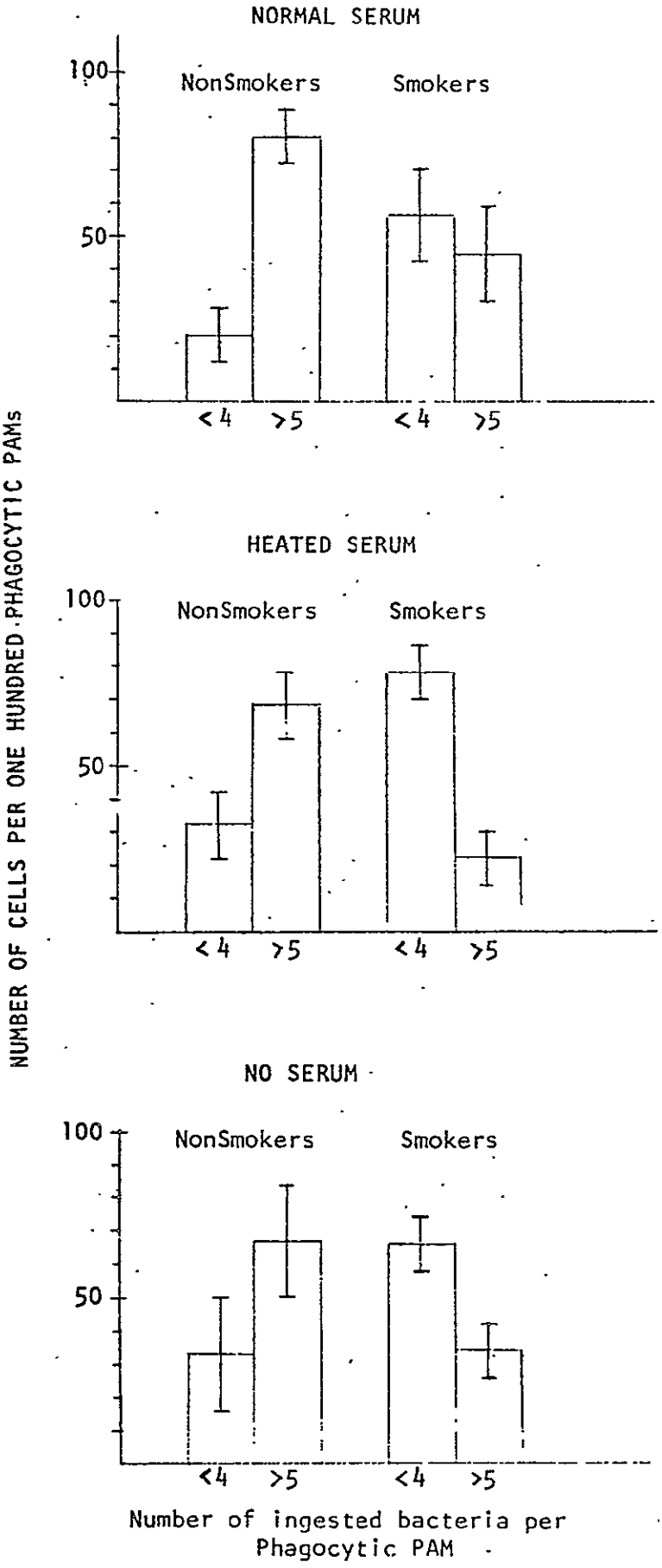

NORMAL SERUM
100
50
NonSmokers
Smokers
<4
>5
<4
>5
HEATED SERUM
100
50
NonSmokers
Smokers
<4
>5
<4
>5
NO SERUM
100
50
NonSmokers
Smokers
<4
>5
<4
>5
NUMBER OF CELLS PER ONE HUNDRED PHAGOCYTIC PAMs
Number of ingested bacteria per Phagocytic PAM

FIGURE 16

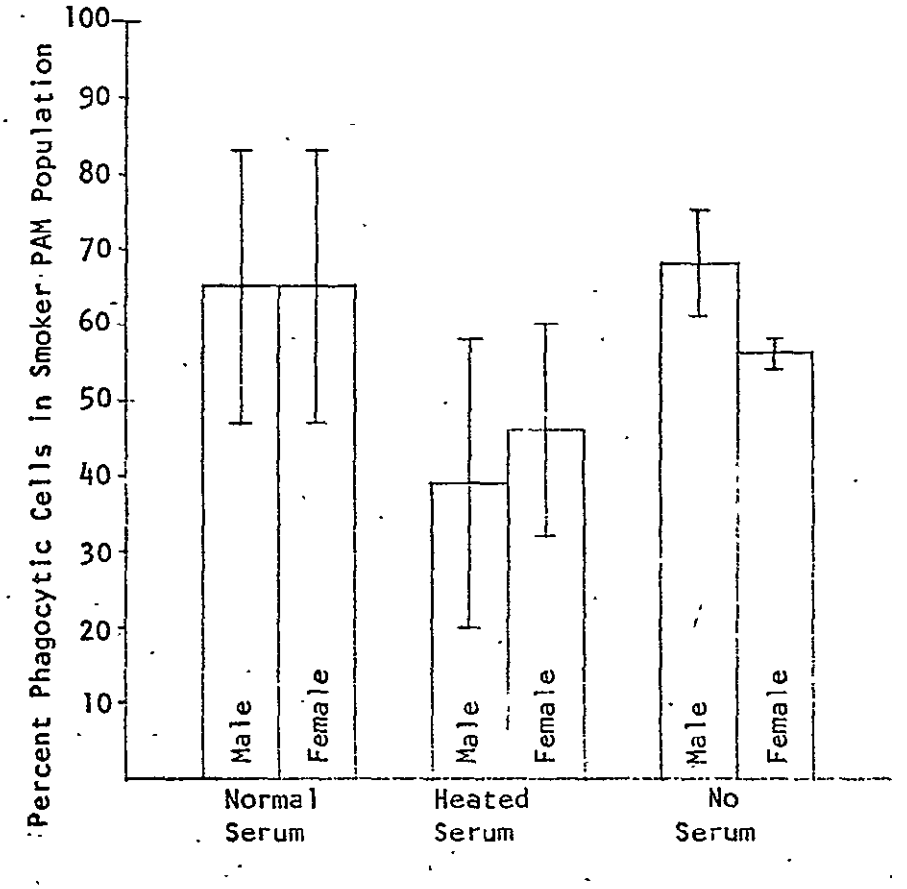
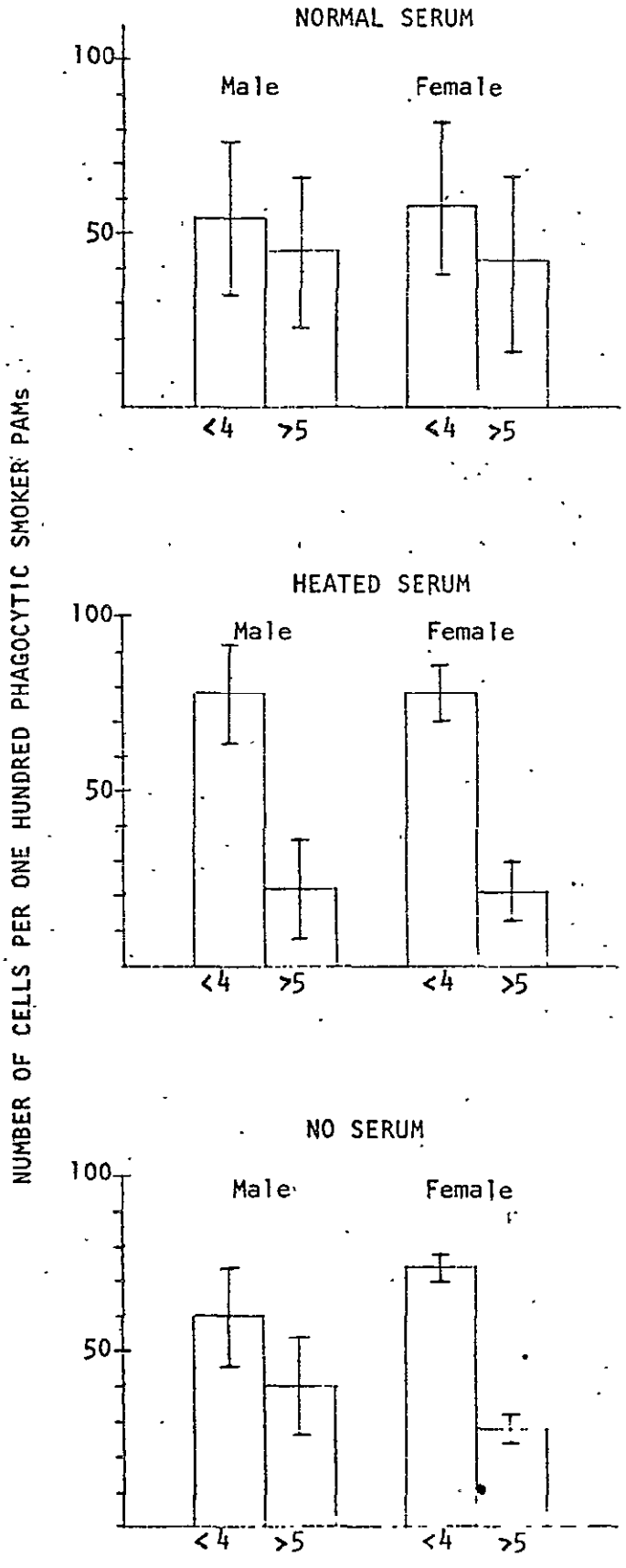

Percent Phagocytic Cells In Smoker PAM Population
100
90
80
70
60
50
40
30
20
10
Male
Female
Male
Female
Male
Female
Normal Serum
Heated Serum
No Serum

FIGURE 17

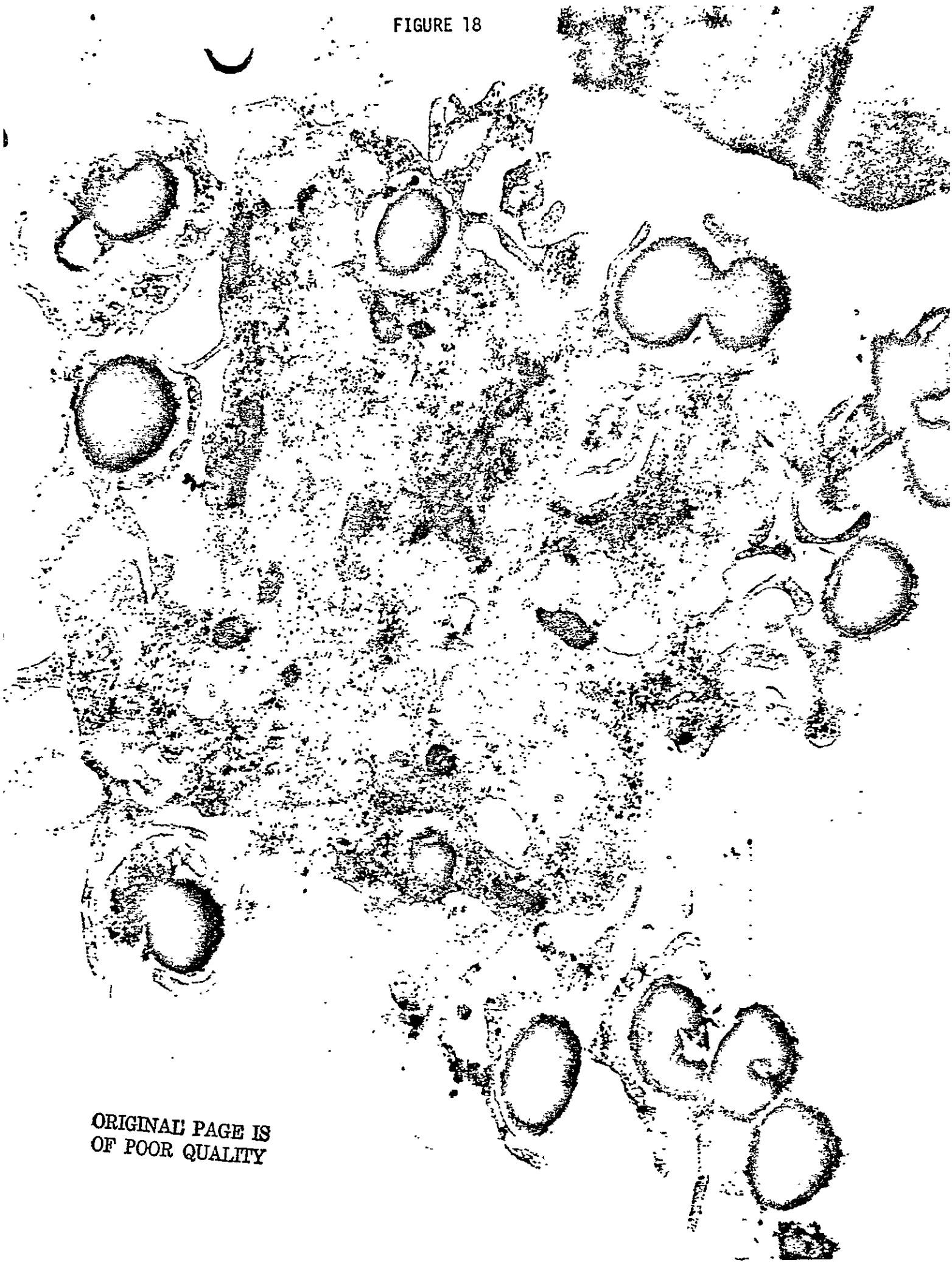

NORMAL SERUM
100
50
Male
Female
<4
>5
<4
>5
HEATED SERUM
100
50
Male
Female
<4
>5
<4
>5
NO SERUM
100
50
Male
Female
<4
>5
<4
>5
NUMBER OF CELLS PER ONE HUNDRED PHAGOCYTIC SMOKER PAMs
Number of ingested bacteria per phagocytic PAM

Analyses of bacterial killing by the PAMs of smokers and nonsmokers are still underway. Preliminary observations show no consistent difference in the labeling of intracellular bacteria by radioactive thymidine when nonsmoker and smoker PAMs are compared. It is probable that bacteria which are ingested by PAMs of smokers are inactivated as promptly as bacteria ingested by nonsmoker PAMs. In terms of clinical significance, the increase in the number of PAMs in smokers may compensate for the reduction in phagocytic abilities of individual cells.

3. Ultrastructural Studies of Phagocytosis: Macrophages obtained from a cigarette smoker and from a nonsmoker were studied using transmission electron microscopy to investigate possible differences in the phagocytosis and digestion of bacteria. After macrophages were collected and suspended in medium 199 supplemented with 10% fresh autologous serum, a culture of Staphylococcus aureus was added in a ratio of 10 bacteria to each phagocyte. The tubes were rotated for 15 minutes and for 1 hour at 37°C. Cells were fixed in buffered 3% glutaraldhyde for 18 hours at 4°C. Cells were post fixed in buffered Osmium for 1 hour then stained with uranyl acetate, embedded in resin, and studied with the electron microscope.

The electron microscopic studies tended to confirm the previous observation made by light microscopy. While a number of different electron micrographs were obtained, only two representative ones are included in this report to illustrate some of the differences observed. The macrophage from the nonsmoker (Figure 18) has an undulating surface membrane which readily encloses the staphylococcal particles. This micrograph shows relatively few of the amorphous cytoplasmic inclusions which are characteristic of the macrophage from the cigarette smoker (Figure 19). In spite of the presence of large amounts of inclusion material, the smoker macrophage is able to form phagocytic vacuoles. Those particles which are ingested undergo deterioration in the same way that particles taken into nonsmoker macrophages are digested. In general, there are fewer organisms taken in by cells from smokers with abundant amounts of cytoplasmic inclusions. More elaborate studies would be required to establish with certainty that the phagocytic vacuoles which are formed are transformed into phagolysosomes in a normal fashion, but the degeneration of staphylococcal particles within the vacuoles seem to progress similarly in cells from smokers and from nonsmokers.

FIGURE 18



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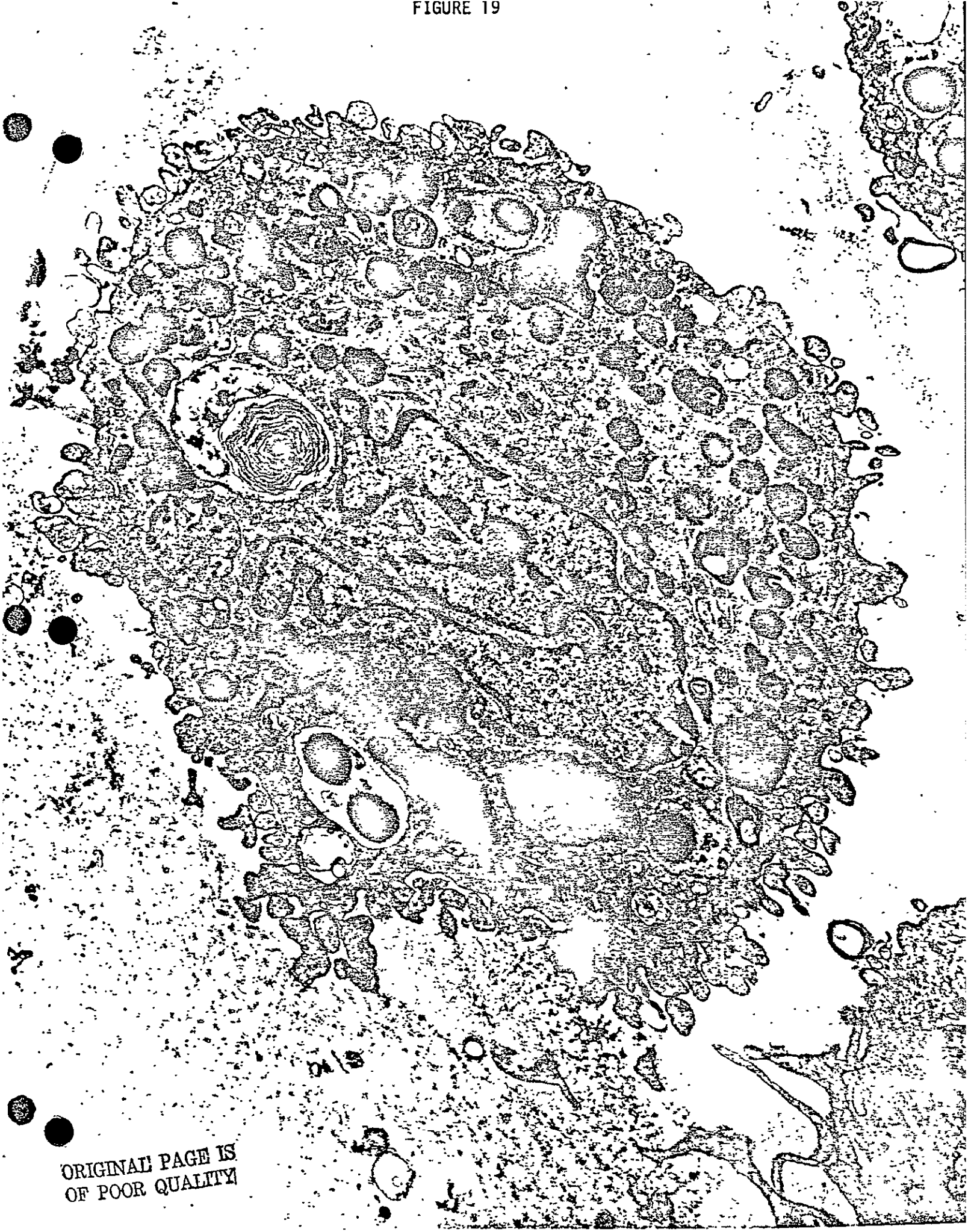
OF POOR QUALITY

FIGURE 19



4. Bronchial Immunoglobulins and Other Proteins: In addition to the cellular changes which accompany exposure of the respiratory tract to cigarette smoke, there are alterations in the proteins present in the lower respiratory tract which may be the result of inflammation from smoking.

In the studies to be described, bronchial lavages were performed on 43 healthy subjects ranging in age from 19 to 46 years (median 25.2 years). Thirty of the subjects (17 males and 13 females) were nonsmokers, while 13 subjects (8 males and 5 females) were cigarette smokers who consumed 20 or more cigarettes daily, with accumulative exposure of from 2 to 9 pack years. The cells obtained by lavage were removed and used in some of the previously described studies. The supernatant fluids were concentrated from 10 fold to 70 fold by a membrane filtration, then stored at -70°C until tested. The concentrated supernatants were tested for immunoglobulins IgG, IgA, IgM, IgD, and IgE using commercially supplied single radial immunodiffusion plates (Meloy). Preliminary experiments were performed using ^{125}I labeled IgG indicated that the immunoglobulin loss from the concentration procedure ranged between 25 and 40% of the immunoglobulin in the original sample. Since these losses might vary from individual to individual, the values presented in this report were not corrected for the protein losses which occurred during concentration.

The observations made on the material lavaged from 13 smokers and 30 nonsmokers is presented in Table IX. The amount of fluid recovered during lavage was similar in the two groups. As emphasized previously, the number of cells was markedly increased in the lavage recovered from cigarette smokers. The total protein (as determined by the Lowery technique) was increased almost 2 fold in the supernatant fluids recovered from smokers. The IgG was also twice as high in the lavage of cigarette smokers (while serum IgG values were no different between the two groups). Finally, in contrast to the upper respiratory tract where IgA predominates, there was less IgA than IgG in the bronchial secretions. The amounts of IgA in both groups of subjects were identical.

These observations indicated that cigarette smoking is associated with an increase in the protein and in IgG in the lower respiratory tract. This could be the result of increased synthesis of proteins by the larger number of lymphocytes and macrophages present in the lungs of smokers, or these changes could result from increased transport of serum proteins into the bronchial tree in response to inflammation accompanying smoking.

In an attempt to determine whether increased exudation of plasma proteins might be responsible for the increases observed in the bronchial washings of cigarette smokers, compared to studies performed on 4 of the serum proteins which are also detectable in bronchial fluids (Table X). While the levels of transferrin and albumin were slightly higher in the fluids from smokers, these differences were not statistically significant and did not settle

TABLE IX

Effects of Cigarette Smoking on Bronchial Proteins

	Nonsmokers (N=30) Mean ± SE	Smokers (N=13) Mean ± SE	P value†
% Lavage return	57.6 ± 2.7	58.6 ± 3.75	>.1
Cells	16.5 ± 2.0 x 10^6	78.9 ± 9.9 x 10^6	<.001
Total Protein*	67.9 ± 6.1	119.36 ± 16.2	<.001
IgG lavage*	6.02 ± 0.5	12.6 ± 2.6	<.001
IgA lavage*	2.06 ± .06	2.03 ± .28	>.9
IgG serum (mg%)	1088.5 ± 32.14	1076.9 ± 65	>.8

* Total mg recovered by lavage
† As determined by T-test

TABLE X

LEVELS OF SELECTED SERUM PROTEINS IN BRONCHIAL LAVAGE FLUIDS
Protein Concentration (mg%)

	Nonsmokers	Smokers	P value
1 antitrypsin	1.45 ± 0.17 mg% (N=33)	1.8 ± 0.32 (N=6)	.9
2 globulin	0.14 ± 0.02 (N=33)	0.18 ± 0.02 (N=6)	.9
Transferrin	2.4 ± 0.25 (N=33)	3.3 ± 0.65 (N=6)	.5
Albumin	19.7 ± 5.8 (N=15)	48.6 ± 20.8 (N=7)	.1

the question about the mechanism involved in increasing the bronchial proteins of smokers.

This led to a more direct approach in quantitation which was performed with 11 volunteers (5 smokers and 6 nonsmokers). Two different preparations of ^{125}I-IgG were employed, both of which had comparable half life in the serum (ranging from 15 to 18 days). At intervals ranging from 1 to 14 days following injection of the ^{125}I-IgG, bronchial lavage was performed and the radioactivity in the lavage material was measured in a dual channel gamma spectrometer. Total protein and immunoglobulin determinations were performed in the concentrated lavage samples. However, because of losses of protein which occurred upon concentration and the fact that immunoglobulin could only be detected in the concentrated samples, an accurate esitmate of the specific activity of the IgG and lavage samples could not be made. The results of these comparative studies are summarized in Table XI. The first three subjects, all nonsmokers, were given 20 μCi of ^{125}I-IgG intravenously and lavaged 14 days later. Radioactivity in the lavage samples was extremely low and was barely detectable above background counts. The next three subjects, all smokers, had an increased dose of 50 μCigiven. This resulted in higher levels of serum radioactivity and increased numbers of counts in the lavage supernatants. The remaining subjects (3 nonsmokers and 2 smokers) were given doses of 100 μCi of ^{125}I-IgG intravenously, and lavages were performed after 24 hours. Serum counts were considerably higher than in the previously tested groups and higher levels of radioactivity were detected in the lavage supernatants of both the nonsmokers and smokers. In spite of comparable serum radioactivity levels, the smokers had a five fold higher level of radioactivity in the lavage fluids. This suggested that transport of serum IgG into the bronchial tree of smokers was increased, and that this might be the mechanism by which cigarette smoking leads to increased protein levels in the bronchial fluids.

5. Complement Activity in Lavage Fluids: Because of the broad range of immunological reactions involving the complement system, pulmonary lavage fluids from 5 nonsmokers and 5 smokers were concentrated 100 fold and one of the complement proteins (C3) was measured (Table XII). The total amount of C3 recovered from the nonsmokers and smokers was comparable. The concentration of this protein in the supernatant fluids from lavage was quite low, acheiving about 10% of normal serum levels even after the supernatants were concentrated 100 fold.

TABLE XI

Levels of Radioactivity Detected in Bronchial Lavage Fluids of Smokers and Nonsmokers After Intravenous ^{125}I-IgG Administration

Dose of ^{125}I-IgG given	Subjects	Counts per minute		
		Lavage	Serum	Ratio
20 µCi	Nonsmokers (N=3)	2.6	1028	.0025
50 µCi	Smokers (N=3)	10.1	2690	.0037
100 µCi	Nonsmokers (N=3)	7.7	36542	.0002
100 µCi	Smokers (N=2)	38	31531	.0012

TABLE XII

COMPLEMENT (C'3) LEVELS IN PULMONARY LAVAGE FLUIDS

	100x (conc) (mean)	Total recovered (mean)
Nonsmokers	12.2 mg%	192 µg
Smokers	11.9 mg%	190 µg

serum C'3 values = 120 mg%

C. Effects of Acute Respiratory Infection:

The preceding sections of this report have documented some of the baseline observations made on materials obtained from normal healthy volunteers who were either nonsmokers or cigarette smokers. Since there are changes in both cells and bronchial proteins associated with cigarette smoking, and since approximately 40% of the adult population are cigarette smokers, it is crucial to determine the changes which must be attributed to smoking before one can interpret the possible changes associated with respiratory infection.

In studying the effects of acute respiratory infection on the cells and proteins obtained by bronchial lavage, it is necessary to initiate studies with comparatively benign infecting agents (such as rhinoviruses) and to be careful that the volunteer does not experience increased morbidity because of the lavage procedure. For these reasons, we have cautiously initiated studies to investigate the effects of viral respiratory infections on the materials obtained by pulmonary lavage.

A total of 4 volunteers have been evaluated by serial bronchial lavage in two separate studies involving experimentally induced rhinovirus infection. The results of these investigations will be summarized in this section.

1. Rhinovirus Study I: Two volunteers with low levels of neutralizing antibody to rhinovirus Type 13 were hospitalized and a baseline bronchial lavage was performed 5 days before intranasal inoculation with Type 13 rhinovirus. Infection with rhinovirus was produced in both volunteers, as evidenced by virus isolation and by increases in serum neutralizing antibody (Table XIII). Only one of the subjects became clinically ill, with the appearance of tracheobronchitis, rhinitis, and sinusitis. The other subject, although infected, did not have symptomatic illness.

The results of the three pulmonary lavages, performed 5 days before virus inoculation and 10 and 17 days after inoculation, are summarized in the following two tables. The volunteer who was infected and who developed clinical illness had an exceptionally large number of cells recovered in the control lavage 5 days before inoculation. Except for an increase in the number of small monocytes (from 5.4×10^6 to 9.3×10^6), there was no significant change in the cell populations obtained at their different sampling times. After illness, a larger proportion of the PAMs contained large amounts of cytoplasmic inclusions (morphologic type 4 cells). No significant differences in acid phosphatase staining were noted when the three samples were compared (Table XIV). The studies performed on the other volunteer, M.W. (who had infection but no illness) also showed little or no changes in the cell population obtained or in the histochemical staining of acid phosphatase when the three time intervals are compared (Table XV).

TABLE XIII: RHINOVIRUS STUDY I (Type 13)

Illness Chart

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
J. D.											TB1			R1 TB2 S1	R1 S1	R1 TB2	TB1	TB1			
M. W.				R1																	

TB-Tracheobronchitis, R-Rhinitis, S-Sinusitis

Virus Isolation from Nasal Secretions

	Days post-challenge							
	-5	1	2	3	4	5	7	8
J. D.	0	0	[+]	+	0	+	[+]	0
M. W.	0	[+]	+	+	+	+	[+]	0

	Nasal Secretory IgA		Serum Neutralizing Antibody*	
	pre-	post-	pre-	post-
J. D.	.205	1.57	4	128
M. W.	.196	0.60	2	64

*16 $TCID_{50}$

TABLE XIV

RHINOVIRUS STUDY: EFFECTS ON PAM

J. D.: 20 year old with infection and clinical illness

	Pre-Challenge	Post-Challenge	
	5-Days	10-Days	17-Days
% Lavage volume returned	60.0	42.0	48.0
Total No. cells recovered	1.80×10^8	1.33×10^8	5.85×10^7
% PAM	91	84	86
% Lymphocytes	5	8	5
% Small-monocytes	3	7	1
PAM "Cell type":			
+1	1	3	0
+2	11	9	3
+3	21	4	8
+4	67	84	89
Acid Phosphatase:			
+1	2	3	2
+2	2	6	4
+3	3	5	6
+4	93	86	88

TABLE XV

RHINOVIRUS STUDY: EFFECTS ON PAM

M. W.: 20 year old with infection but no illness

	Pre-Challenge	Post-Challenge	
	5-Days	10-Days	17-Days
% Lavage volume returned	60.0	72	56.0
Total No. cells recovered	9.2×10^7	1.08×10^8	1.12×10^8
% PAM	88	93	77
% Lymphocytes	8	6	14
% Small-monocytes	3	1	1
PAM "cell type":			
+1	6	3	3
+2	15	8	14
+3	10	13	14
+4	69	76	69
Acid Phosphatase:			
+1	1	0	5
+2	4	2	5
+3	9	8	11
+4	86	90	79

The in vitro phagocytic function of the macrophages obtained from these two volunteers was tested, using the previously described autoradiographic method. The studies with PAMs from the subjects who became ill (Table XVI) and the volunteer who was infected but not clinically ill (Table XVII) showed preservation of phagocytosis of staphylococci, as well as maintenance of staphylococcal inactivation as measured by the thymidine uptake of intracellular bacteria. If any effect of the viral infection on phagocytic function of PAMs occurred, there may have been improvement in phagocyte function (rather than impairment) when the post infection cells are compared to the control cells.

The supernatant fluids obtained from the three lavages on each subject were concentrated 10 fold and immunoglobulins and other proteins were measured as before (Table XVIII and XIX). Except for the presence of higher levels of IgG, alpha$_1$ antitrypsin, alpha$_2$ macro globulin, transferrin, and albumin in the concentrated lavage fluids of the subjects who became ill (Table XVIII) when compared to the values in the lavage fluids of the subjects who was infected but not ill (Table XIX). The IgA levels in the bronchial fluid rose after infection then returned toward normal in the subjects without illness, while little or no elevation occurred in the ill subject. To attempt to relate these differences in IgA responsiveness to the occurrence of illness would be entirely speculative, but such a relationship could conceivably exist if IgA is an important defense mechanism in the lower respiratory tract, as it seems to be in other body fluids.

2. Rhinovirus Study II: Volunteers with low levels of neutralizing antibody to rhinovirus were inoculated intranasally with rhinovirus Type 13 and serial bronchial lavages were performed. As in the previous study, only one of the two individuals became clinically ill, although both volunteers became infected with the inoculated rhinovirus (Table XX). Both subjects has a rise in neutralizing antibody to the infecting organism, and both had increases in the amount of IgA obtained in nasal secretions. The lavage intervals were slightly different in this study than in the previous rhinovirus study, with the control lavage coming two days before inoculation and the post inoculation lavages occurring at 14 and 21 days after infection. One of the volunteers for this study was a cigarette smoker, while the other was a nonsmoker. The cells obtained from the control bronchial lavage two days before inoculation were within the range expected for the smoking status of the individual volunteer (Table XXI). An increase in the number of cells, particularly PAMs and lymphocytes, occurred in the smoker who became clinically infected. More modest elevations occurred in the specimen obtained 14 days after inoculation from the subject who became infected but not ill.

TABLE XVI: RHINOVIRUS STUDY: EFFECTS ON PAM PHAGOCYTOSIS

J. D.: Cigarette Smoker with 5 pack-years Age: 20, Male

	5 days pre-challenge						10 days post-challenge						17 days post-challenge					
	No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum	
Phagocytosis time interval (Min)	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30
% Phagocytic PAM	80	90	44	48	76	92	100	100	49	60	59	78	99	100	97	98	98	95
% Phagocytic PAM with <4 ingested bacteria	15	13	24	24	28	11	6	0	35	31	23	28	7	0	4	2	6	9
% Phagocytic PAM with >5 ingested bacteria	65	77	20	24	48	81	94	100	14	29	36	50	92	100	93	96	92	86
% Ingested bacteria labeled with ^{3}H-Thymidine	2.2	21	11.9	17.3	20.3	9.5	53.2	23.8	24.9	1.4	28.4	15.5	30.3	44.5	21.8	15.1	66.4	23.2
% Intracellular bacteria killed	97.8	79	88.1	82.7	79.8	90.5	46.8	76.2	75.1	98.6	71.6	84.5	69.7	55.5	78.2	84.9	33.6	76.8

TABLE XVII: RHINOVIRUS STUDY: EFFECTS ON PAM PHAGOCYTOSIS

M. W.: Cigarette Smoker with 11 pack-years Age: 21, Male

	5 days pre-challenge						10 days post-challenge						15 days post-challenge					
	No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum	
Phagocytosis time interval (Min)	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30
% Phagocytic PAM	69	82	26	14	24	20	100	100	81	88	67	77	99	98	73	82	89	89
% Phagocytic PAM with <4 ingested bacteria	23	30	16	11	15	11	0	1	38	36	34	27	1	7	7	16	17	11
% Phagocytic PAM with >5 ingested bacteria	46	52	10	3	9	9	100	99	43	52	33	50	98	91	66	66	72	78
% Ingested bacteria labeled with ^{3}H-Thymidine	53.6	22.0	16.2	10.8	15.6	12.0	42.5	-	18.8	11.2	12.9	6.4	32	52.6	28.7	27	45.9	48.6
% Intracellular bacteria killed	46.4	78	83.8	89.2	84.4	88.0	57.5	-	81.2	88.8	87.1	93.6	68	47.4	71.3	73	54.1	51.4

TABLE XVIII

RHINOVIRUS STUDY: EFFECTS ON SERUM AND LAVAGE PROTEINS

J. D.

Serum (values in mg%)

	γA	γG	γM	α_1 Anti	α_2 Mac	Trans	Alb	γA/Alb	γG/Alb
5 days pre-challenge	195	1500	163	203	282	312	3899		
10-days post-challenge	211	1410	159	226	282	332	3560		
17-days post-challenge	221	1500	167	188	297	344	3729		
Lavage (concentrated 10x, values in mg%)									
5-days pre-challenge	2.2	11.3	-	2.5	0.2	5.0	40.4	0.05	0.27
10-days post-challenge	2.9	11.6	-	2.4	0.3	5.7	43.1	0.06	0.26
17-days post-challenge	1.3	10.4	-	1.4	-	3.4	25.6	0.05	0.40

TABLE XIX

RHINOVIRUS STUDY: EFFECTS ON SERUM AND LAVAGE PROTEINS

M. W.

Serum (values in mg%)

	γA	γG	γM	α1 Anti	α2 Mac	Trans	Alb	γA/Alb	γG/Alb
5-days pre-challenge	211	1500	184	188	185	270	4746		
10-days post-challenge	213	1515	191	199	178	274	4859		
17-days post-challenge	205	1500	190	192	161	293	4690		
Lavage (concentrated 10x, values in mg%)									
5-days pre-challenge	2.4	8.2	-	1.2	-	2.4	19	0.12	0.43
10-days post-challenge	4.2	8.9	-	0.8	-	2.5	17	0.24	0.52
17-days post-challenge	3.5	13.8	-	1.4	-	3.9	34	0.10	0.40

TABLE XX

RHINOVIRUS STUDY II (Type 13)

Illness Chart

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D. H.								R1	R2	R1	R1					
C. W.																

R-Rhinitis

Virus Isolation from Nasal Secretions

	Days post-challenge							
	-5	1	2	3	4	5	7	8
D. H.	0	0	[+]	+	+	+	[+]	0
C. W.	0	0	[+]	+	+	[+]	0	0

Antibody Responses

	Nasal Secretory IgA		Serum Neutralizing Antibody *	
	pre-	post-	pre-	post-
D. H.	0.14	2.57	<2	64
C. W.	0.097	2.35	<2	8

*16 $TCID_{50}$

TABLE XXI

RHINOVIRUS STUDY: EFFECTS ON PAM

D. H.: Smoker

	Pre-Challenge	Post-Challenge	
	2-Days	14-Days	21-Days
% Lavage volume returned	56.0	68.0	62.0
Total No. Cells recovered	3.68×10^7	13.5×10^7	13.6×10^7
% PAM	89.5	87.5	77.5
Total No. PAM	3.29×10^7	11.8×10^7	10.5×10^7
% Lymphocytes	3.5	5.0	8.0
Total No. Lymphocytes	1.3×10^6	6.7×10^6	1.1×10^7
% small-monocytes	7.0	8.0	14.5
Total No. monocytes	2.6×10^6	1.1×10^7	2.0×10^7
Williams, C: Nonsmoker			
% Lavage volume returned	64.0	60.0	62.0
Total No. Cells recovered	1.36×10^7	4.2×10^7	2.2×10^7
% PAM	66.5	78	80
Total No. PAM	9.0×10^6	3.3×10^7	1.8×10^7
% Lymphocytes	26.0	18	17
Total No. Lymphocytes	3.5×10^6	7.6×10^6	3.7×10^6
% small-monocytes	7.5	4	3
Total No. monocytes	1.0×10^6	1.7×10^6	6.6×10^5

The PAMs obtained by pulmonary lavage in the control and the two post illness periods were studied for their ability to ingest and kill staphylococci (Table XXII and XXIII). As in the previous study, there were no consistent changes observed in the phagocytic capacities of PAMs after rhinovirus infection. The bronchial proteins in lavage supernatants from these two subjects were not studied in detail.

Leukocytes were collected from both of the volunteers in this study, and in vitro phagocytosis staphylococci was evaluated in a fashion similar to the test done with PAMs. Blood samples were obtained two days before virus inoculation, and six days, 12 days, and 19 days after infection with rhinovirus. The uptake of staphylococci was quantitated after five, 10, and 15 minutes of incubation. The percentage of phagocytic leukocytes was determined, and the number of bacteria within phagocytes was tabulated. A significant increase was noted in the phagocytosis of staphylococci by leukocytes obtained 6 and 12 days after inoculation from the ill subjects (Table XXIV), while no such increase occurred in leukocytes from the subject who was infected but not ill (Table XXV). The increases that occurred in phagocytosis of staphylococci by leukocytes 6 and 12 days after infection are more apparent when the post infection results are expressed as a ratio of the control observations (Table XXVI).

These observations suggest that infection with rhinovirus does not produce a demonstrable impairment of in vitro staphylococcal phagocytosis, under the conditions studied. In fact, an increase in bacterial phagocytosis may follow this form of viral infection.

TABLE XXII

RHINOVIRUS STUDY II: EFFECTS ON PAM

D. H.: Smoker, Male

	2 days pre-challenge						14 days post-challenge						21 days post-challenge					
	No	Serum	Heated	Serum	Normal	Serum	No	Serum	Heated	Serum	Normal	Serum	No	Serum	Heated	Serum	Normal	Serum
Phagocytosis time interval (Min)	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15
% Phagocytic PAM	60	83	58	84	95	97	51	87	84	97	99	100	51	96	45	82	50	77
% Phagocytic PAM with <4 ingested bacteria	66.7	41.0	12.1	8.3	6.3	1.0	72.5	27.6	3.6	3.1	4	1	27.5	4.2	35.6	8.5	38.0	13.0
% Phagocytic PAM with >5 ingested bacteria	33.3	58.0	87.9	91.7	93.7	99.0	27.5	72.4	96.4	96.9	96.0	99.0	72.5	95.8	64.4	91.5	62.0	87.0
% Ingested bacteria labeled with ^{3}H-Thymidine	38	29	24	25	44	33	38	44	19	29	11	10	25	25	10	24	22	34
% Intracellular bacteria killed	62	71	76	75	56	67	62	36	81	71	89	90	75	75	90	76	78	66

TABLE XXIII: RHINOVIRUS STUDY II: EFFECTS ON PAM

.C. W.: Nonsmoker, Male

	2 days pre-challenge						14 days post-challenge						21 days post-challenge					
	No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum		No Serum		Heated Serum		Normal Serum	
Phagocytosis time interval (Min)	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15	5	15
% Phagocytic PAM	79	97	73	91	57	94	66	86	76	98	93	97	65	90	53	94	77	98
% Phagocytic PAM with <4 ingested bacteria	32.9	11.3	12.3	1.1	38.6	2.1	34.8	16.3	0	4.1	4.3	2.0	21.5	11	24.5	5.3	14.3	3.1
% Phagocytic PAM with >5 ingested bacteria	67.1	88.7	87.7	98.9	61.4	97.9	65.2	83.7	100	95.9	95.8	98.0	78.5	89	75.5	94.7	85.7	96.9
% ingested bacteria labeled with ^{3}H-Thymidine	17	22	12	42	3	25	24	24	17	16	36	40	31	19	18	6	37	21
% Intracellular bacteria killed	83	78	88	58	97	75	76	76	83	84	64	60	69	81	82	94	63	79

TABLE XXIV

RHINOVIRUS STUDY II: EFFECTS ON LEUKOCYTE PHAGOCYTOSIS

Subject: D. H. Smoker, Male

Date	Bacterial ingestion period(min.)	%Leukocytes phagocytic	No. PMN-L with "x" No. ingested bacteria per 100 PMN-L				per 100 phagocytic PMN-L			
			"x"No. bact/PMN-L				"x"No. bact/PMN-L			
			0	1-4	5-10	11+	0	1-4	5-10	11+
2 days pre-challenge	5	19	81	6	8	5	426	31.6	42.1	26.3
	10	21	79	8	9	4	376	38.1	42.8	19.0
	15	40	60	17	14	9	150	42.5	35.0	22.5
6 days post-challenge	5	43	57	13	23	7	132	30.2	53.5	16.3
	10	65	35	12	25	28	53.8	18.5	38.5	43.1
	15	72	28	10	20	42	38.9	13.9	27.8	58.3
12 days post-challenge	5	31	69	14	11	6	222	45.2	35.5	19.4
	10	50	50	12	15	23	100	24.0	30.0	46.0
	15	74	26	11	12	51	35.1	14.8	16.2	68.9
19 days post-challenge	5	11	89	9	2	0	809	81.8	18.2	0
	10	29	71	14	13	3	244	48.2	44.8	10.3
	15	28	72	13	9	11	257	46.4	32.1	39.2

* Cultured in McCoy's 5A medium with 10% heated autologous serum

TABLE XXV

RHINOVIRUS STUDY II: EFFECTS ON LEUKOCYTE PHAGOCYTOSIS

Subject: C. W. Nonsmoker, male

Date	Bacterial ingestion period(min.)	%Leukocytes phagocytic	No. PMN-L with "x" No. ingested bacteria per 100 PMN-L				per 100 phagocytic PMN-L			
			"x"No.bact/PMN-L				"x"No.bact/PMN-L			
			0	1-4	5-10	11+	0	1-4	5-10	11+
2 days pre-challenge	5	38	62	19	17	2	163	50.0	44.7	5.3
	10	53	47	27	22	4	88.6	50.9	41.5	7.5
	15	63	37	24	17	22	58.7	38.0	26.9	34.9
6 days post-challenge	5	37	63	21	12	4	170	56.7	32.4	10.8
	10	40	60	13	12	15	150	32.5	30.0	37.5
	15	53	47	13	19	21	88.6	24.5	35.8	39.6
12 days post-challenge	5	21	79	5	8	8	376	23.8	38.0	38.0
	10	43	57	2	21	20	132	4.6	48.8	46.5
	15	53	47	7	14	32	88.6	13.2	26.4	60.3
19 days post-challenge	5	17	83	9	4	4	488	52.9	23.5	23.5
	10	30	70	14	9	7	233	46.6	30.0	23.3
	15	60	40	13	26	21	66.6	21.6	43.3	35.0

* Cultured in McCoy's 5A Medium with 10% heated autologous serum

TABLE XXVI

RHINOVIRUS STUDY II: EFFECTS ON LEUKOCYTE PHAGOCYTOSIS

	Date	%PMN-L Actively Phagocytic*	Ratio to pre-challenge	%Phagocytic PMN-L with 11 ingested bact.	Ratio to pre-challenge
(smoker)	2 days pre-challenge	40		22.5	
	6 days post-challenge	72	1.8:1	58.3	2.59:1
	12 days post-challenge	74	1.85:1	68.9	3.06:1
	19 days post-challenge	28	0.7:1	39.2	1.74:1
C. W. (nonsmoker)	2 days pre-challenge	63		34.9	
	6 days post-challenge	53	0.84:1	39.6	1.1:1
	12 days post-challenge	53	0.84:1	60.3	1.7:1
	19 days post-challenge	60	1:1	35.0	1:1

* Bacterial ingestion period of 15 min. Incubated in McCoy's 5A Medium with 10% heated autologous serum.

Staphylococcal-PMN-L ratio of 20:1

II. Polymorphonuclear Leukocytes

A. General Observations:

Samples of the peripheral blood are readily obtainable, and may be studied serially for changes which occur in the cells or in the plasma. Because the polymorphonuclear leukocytes remain in the circulation for a matter of a few hours after they are released, changes which occur in these cells might be expected to reflect alterations in the host which occur in association with illness or disease. Emphasis in the past has been placed on alterations and total and differential white blood cell counts in diagnosing acute infections, particularly those caused by bacteria. This section will summarize some of the observations made using polymorphonuclear leukocytes (PMNs) as a possible approach to the early detection of disease. A variety of morphological, histochemical, and functional observations have been made which will provide background information for possible development of tests employing PMNs in the laboratory tests for early detection of disease.

B. Studies of Normal Controls:

In many of the studies employing relatively large numbers of leukocytes, such as phagocytosis and chemotaxis testing, leukocytes must be separated from the whole blood and suspended in the desired concentration for the test to be performed. A widely used method of separating leukocytes from the whole blood, and the one usually employed in our laboratory, is the addition of blood to seditmentation fluid containing dextran and an anticoagulant. The erythrocytes agglutinate and settle rapidly, leaving leukocytes and platelets in the plasma phase. This separation procedure vastly reduces the number of erythrocytes, without appreciably changing the distribution of the various leukocytes left behind. Some leukocytes are trapped among the settling erythrocytes, a loss which is usually in the order of 40% (Table XXVII). These semipurified leukocytes can then be employed in the desired *in vitro* tests. The observation shown in this table represent recovery of leukocytes from 12 different normal volunteers, and they are representative of the results obtained from a much larger overall experience with this sedimentation technique.

It has become apparent that any technique which is to be considered as a test for illness or disease must be reproducible within acceptable limits, so that a reasonable deviation from the normal range will reflect changes in the cells rather than just fluctuations in technique. As reported in the annual report from the first year of this project, we have set up several different tests employing histochemical staining, *in vitro* migration, and phagocytic capacity of PMNs to evaluate their usefulness in disease detection. Eight volunteers (4 males and 4 females) were tested on the same day each week over an eight week period, and a variety of observations was made. These studies have just been

TABLE XXVII

DEXTRAN SEDIMENTATION OF LEUKOCYTES FROM WHOLE BLOOD - EVALUATION

Recovered Leukocyte Differential (Wright-Giemsa Stain)

% Total WBC recovered	Lymphocytes	Neutrophiles	Eosinophiles	Basophiles	Monocytes
68.8	39	45	3.5	0	12.5
33:7	31	61.5	5.5	0	2.0
77.7	37.5	57.0	2.5	.5	2.5
85.3	28.3	65.7	4.0	1.0	1.5
47.8	33	60.0	2.5	2.5	2.5
58.2	27.5	65.0	4.5	0.5	2.5
54.4	21.0	77.0	0.5	0.5	1.0
68.0	28.0	67.5	3.0	0	1.5
73.0	20.2	73	3.0	1.0	2.5
59.2	22.6	72.7	0	0	5.0
53.8	16.4	82.0	.5	0	1.5
48.0	16.3	81.0	1.0	.5	2.0
mean 60.6	26.8	67.2	2.5	0.5	3.0
std. error 4.0	2.1	2.9	.5	.2	.9
Normal values of whole blood:	25.0	65.0	4.0	<1.0	3.0

completed, but the data is still being analyzed. Some of the initial observations are presented in this section.

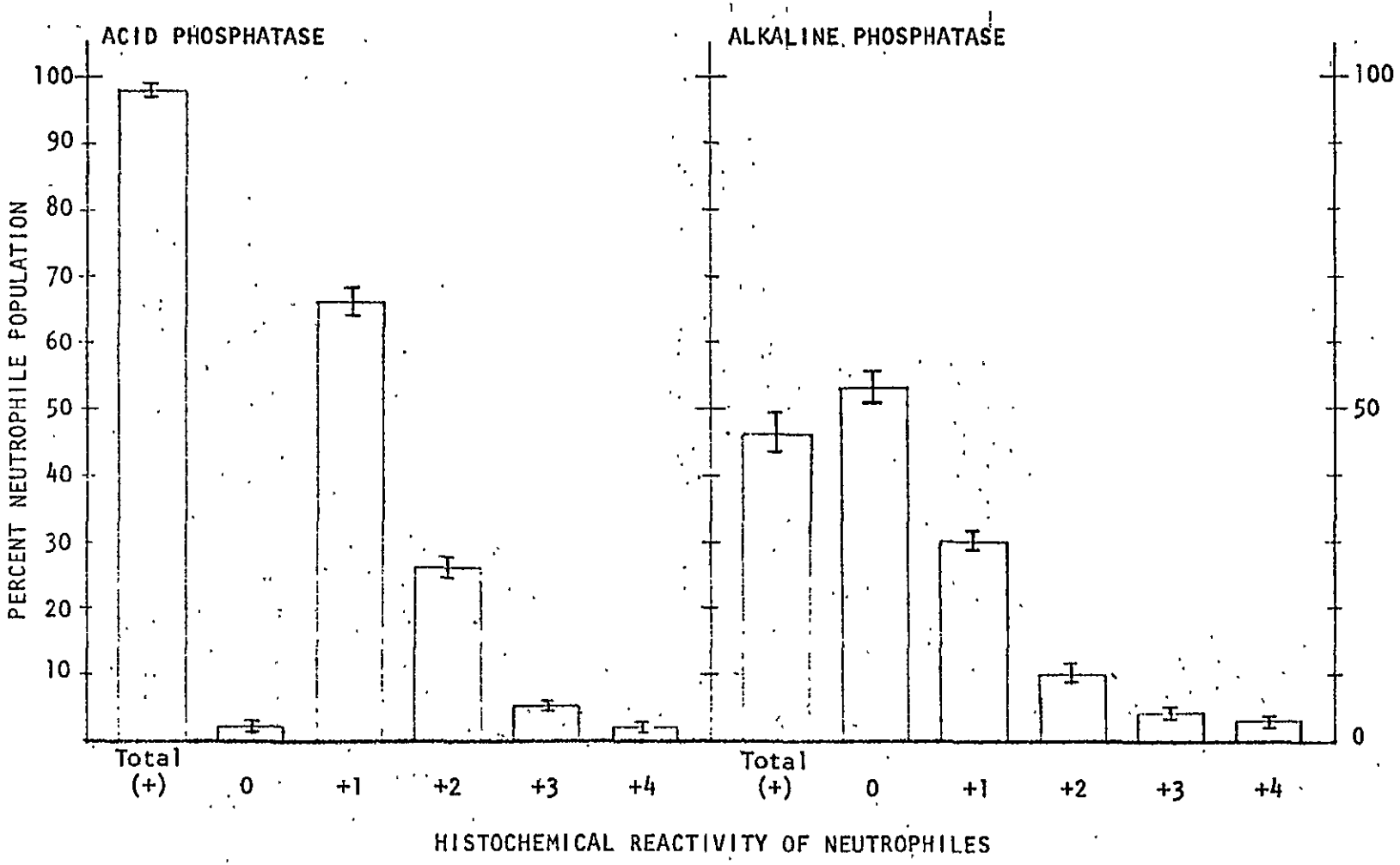
Peripheral blood before dextran sedimentation was used to prepare thin smears for histochemical staining. These were processed for acid phosphatase and alkaline phosphatase, and the intensity of histochemical staining of individual leukocytes from each blood sample was evaluated. In these normal subjects, essentially all of the PMNs had some reactivity for acid phosphatase, but most of these cells were only weakly reactive (Table XXVIII). Over half of the cells from the same samples stained for alkaline phosphatase showed no reaction, and very few of these cells showed intense staining. When the total white blood cell counts and differential counts on the same samples of blood are employed, these percentages can be used to calculate the number of cells in each blood sample with each level of staining intensity (Table XXVIX). When the planned 8 week study is finished, we should have a better understanding about the variation from sample to sample in the values observed in normal healthy individuals.

One of the in vitro functions of PMNs that we have studied most intensively is migration of these cells through micropore filters. Migration can be studied in medium alone (random migration), and chemotactic responsiveness to a number of different attracting substances can be evaluated. Leukocytes from the same 8 volunteers mentioned above were studied in modified Boyden chambers. Random migration, and chemotactic responsiveness to casein (100 mg%) and to A_2 Hong Kong Virus (16 chick cell agglutinating units/ml) were evaluated weekly for 8 weeks. Casein proved to be a satisfactory and reproducible chemotactic substance, resulting in approximately ten times as many cells migrating through the filter when casein was used as when no attracting substance was present. The influenza virus, however, was a much weaker chemotactic agent, resulting in between 2 and 3 fold increases over random migration with leukocytes from most individuals (Figure 20).

In order to study the variables which might be important in further modifying the chemotaxis assay, a small number of studies were done in which the number of leukocytes placed in each chemotaxis chamber was varied. Over the range of leukocytes employed (from 3 times 10^5 to 6 x 10^6 WBC/chamber) there was a linear relationship between the WBC's added and the PMNs migrating through the filter (Figure 21). This suggests that it may be possible to reduce the number of PMNs required to perform an individual assay, making the blood requirements for performing the test less demanding.

An additional functional property of PMNs which is important and which may be modified by infection or disease is phagocytosis of foreign particles. Repeated studies were done on the same group of normal individuals in which the phagocytosis of staphylococci

TABLE XXVIII



Normal controls of histochemical reactivity of neutrophiles in whole blood smears

TABLE XXIX

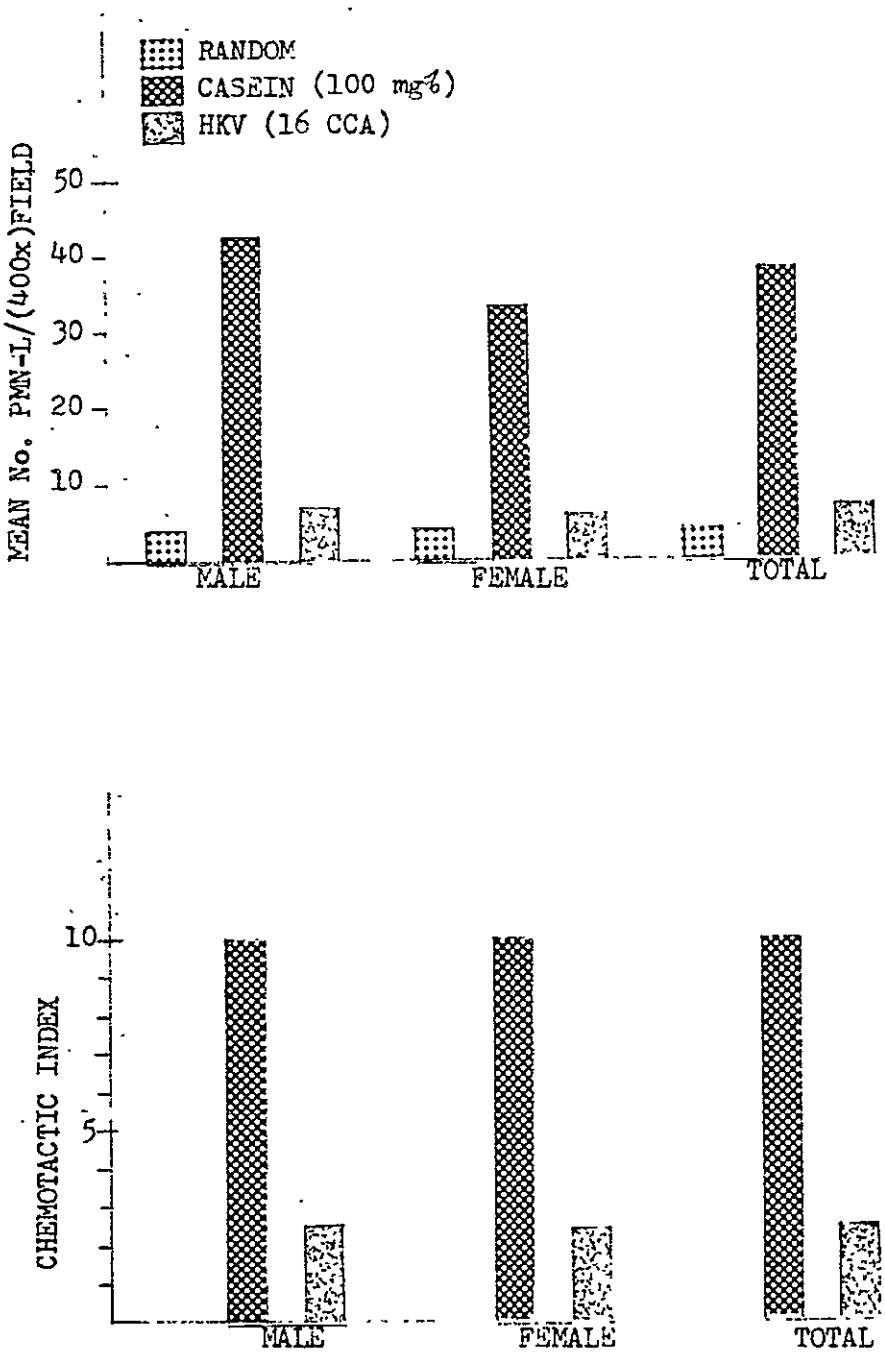
HISTOCHEMISTRY REACTIVITY OF NEUTROPHILES
(NORMAL CONTROLS)

	# Neut./mm^3 blood	# Neut./mm^3 - Acid Phosphatase Reactivity					
		0	+1	+2	+3	+4	Total (+)
mean	3044.0	64.2	1996.8	767.3	144.0	69.6	2977.6
std. error	209.7	28.7	166.7	100.6	23.1	20.6	199.5

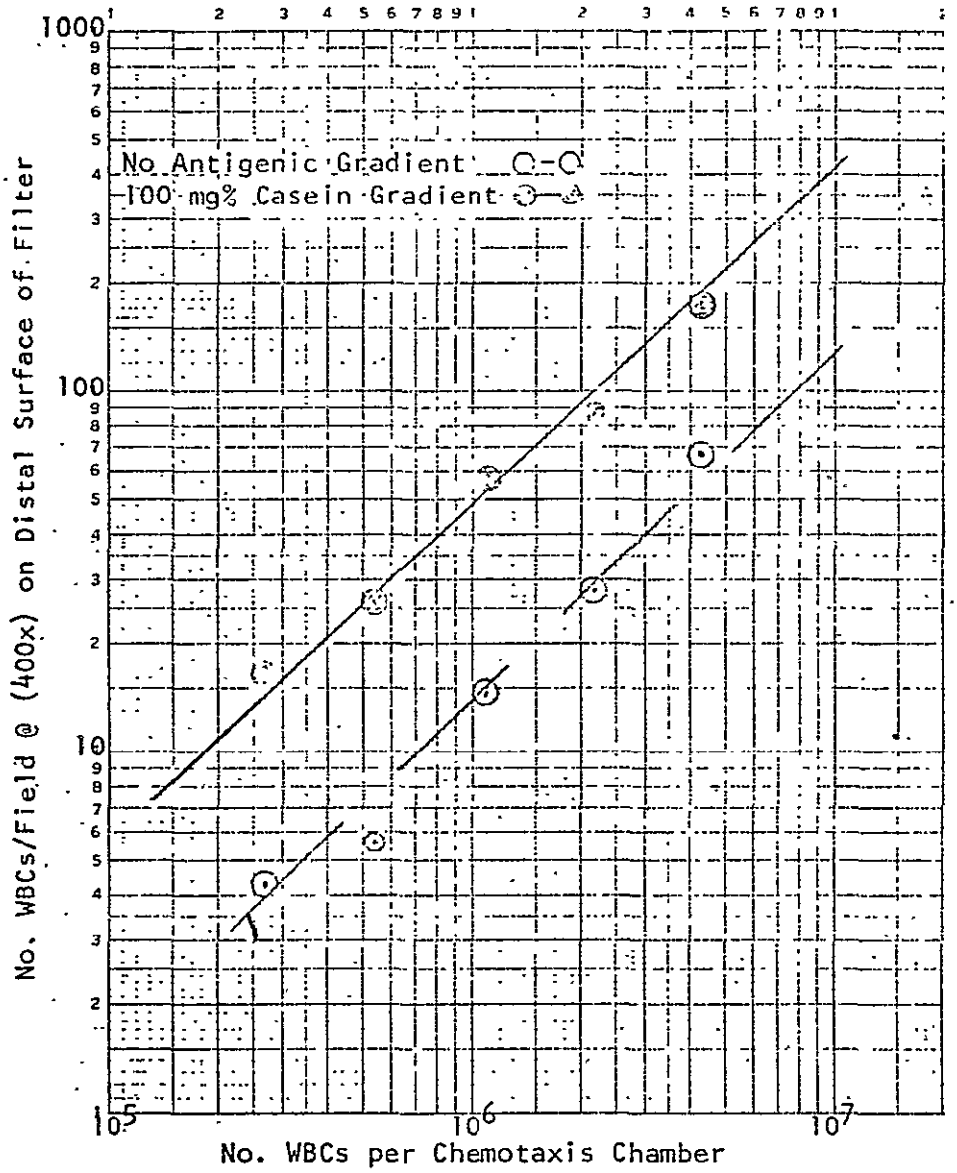
	# Neut./mm^3 - Alk. Phosphatase Reactivity					
	0	+1	+2	+3	+4	Total (+)
mean	1582.1	938.7	300.6	126.9	95.7	1461.8
std. error	126.1	126.1	48.8	29.6	18.4	165.7

N = 29

FIGURE 20



CHEMOTAXIS CONTROL STUDY: Test conditions in TRIS ACM with 10% fresh autologous serum at weekly intervals for two to four weeks. RANDOM - no antigenic gradient, ANTIGEN STIMULANTS - casein at 100 mg%, and A_2 Hong Kong virus at 16 CCA Units/ml, on distal side of 3um porosity filter, incubated at 37°C for 2-1/2 hours. Males, n=5; Females, n=4; Total, n=9


No Antigenic Gradient C-C
100 mg% Casein Gradient
No. WBCs/Field @ (400x) on Distal Surface of Filter
No. WBCs per Chemotaxis Chamber
1000
100
10
10^5
10^6
10^7

was quantitated microscopically. Data has been compiled on some of the initial observations, and the phagocytosis assay seems to give acceptable reproducibility (Figure 22). PMNs were incubated for varying lengths of time (3, 6, 10, and 15 minutes), then the slides were fixed and stained, and the percent of PMNs which were phagocytic was determined microscopically. No significant difference was noted between leukocytes from the healthy male or female volunteers. Under the conditions of the assay, over 90% of the PMNs contained at least one staphylococcus after 15 minutes. The number of organisms within each phagocytic cell increased steadily as the time of incubation increased, so that half of the phagocytes contained 11 or more bacteria in 10 minutes, with a further increase to over 70% of the phagocytes with 11 or more bacteria by 15 minutes (Figure 23). The range of variation was acceptably small in repeated observations using leukocytes from the same person, so continued studies of the uptake of bacteria are indicated, since one can expect that this system could have the ability to detect alterations in phagocytosis if such changes occur as one of the manifestations of disease.

C. Effects of Acute Respiratory Infection:

Several small pilot studies have been performed using leukocytes obtained from the volunteers in the respiratory virology studies conducted by Drs. Robert Couch, Julius Kasel, Vernon Knight, and Stephen Greenberg in the Department of Microbiology and Immunology. Leukocytes have been obtained from small groups of volunteers with influenza, rhinovirus, and Mycoplasma pneumoniae infections, experimentally induced in susceptible volunteers. Leukocytes have also been collected and studied from volunteers with a variety of naturally occurring acute respiratory infection syndromes. The results of these PMN studies are currently being compiled, and the data will be analyzed in conjunction with the observations on lymphocytes from the same subjects made in Dr. Criswell's laboratory The results of these studies will be included in future progress reports.

LEUKOCYTE - STAPHLOCOCCAL PHAGOCYTOSIS
(NORMAL CONTROLS)

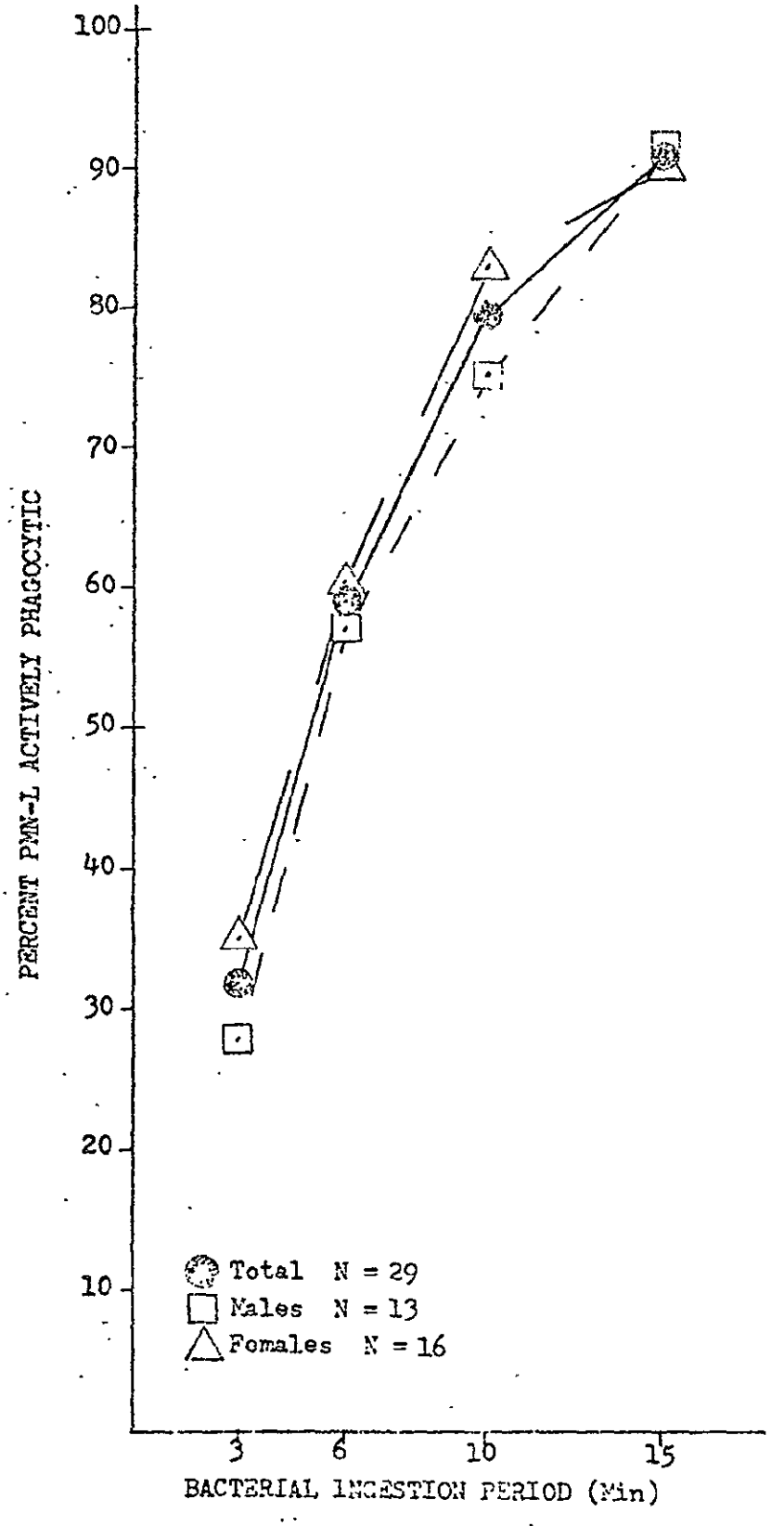
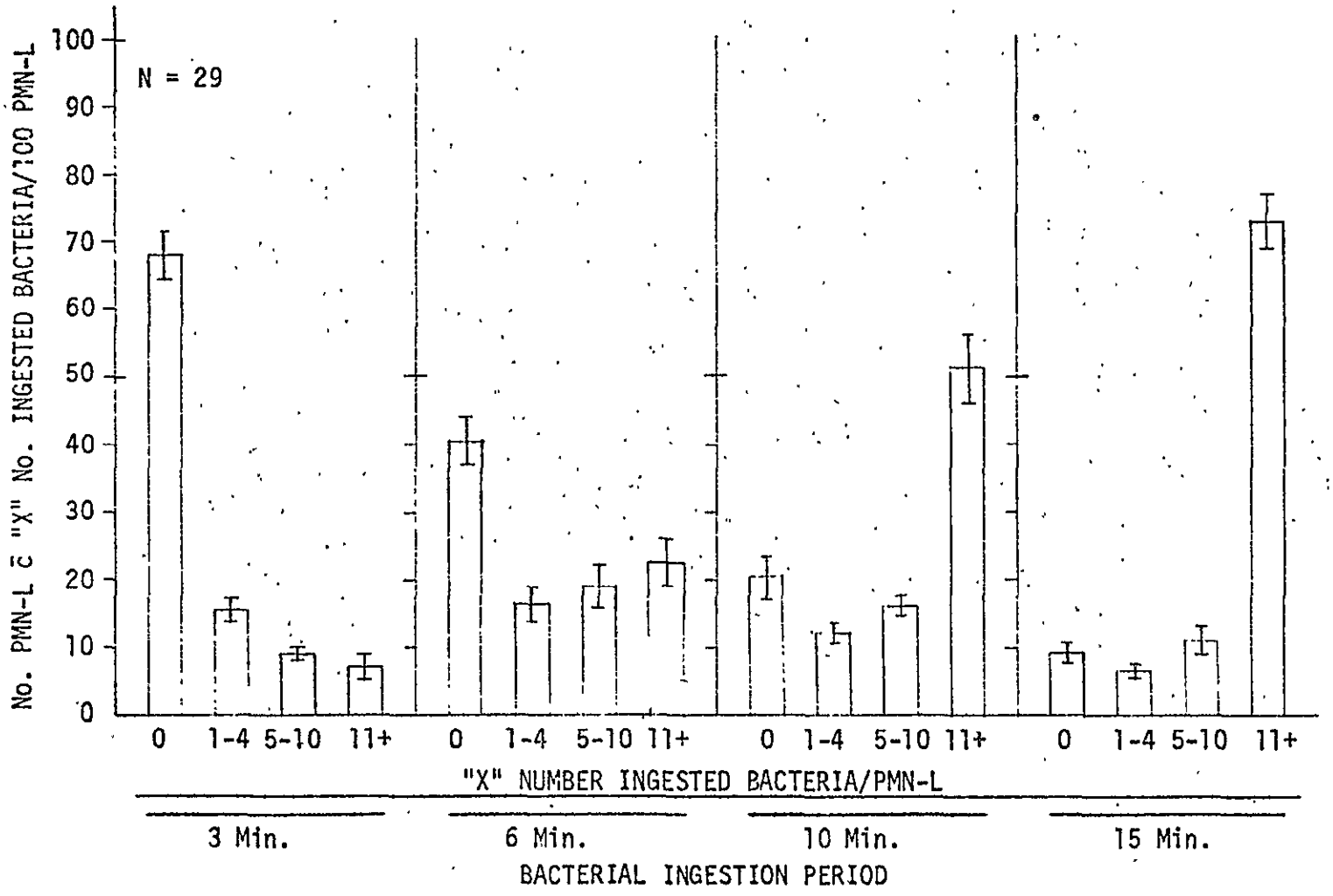


FIGURE 23: LEUKOCYTE - STAPHLOCOCCAL PHAGOCYTOSIS (NORMAL CONTROLS)



McCoy's 5A Medium with 10% serum

III. A Developing Perspective

The studies during the past two years of this project have utilized pulmonary alveolar macrophages (PAMs) and polymorphonuclear leukocytes (PMNs) to investigate possible changes in morphology or function of these cells associated with disease, especially acute respiratory infection. The studies involving PAMs have raised fascinating questions concerning the possible significance of the alterations produced by cigarette smoking in otherwise healthy persons. It has become apparent that the abnormalities which occur in the PAMs due to cigarette smoking (a practice which is so prevalent that any useful tests will have to be applicable to cells from smokers) make it unlikely that the use of these cells will give a clear cut answer in the early detection of disease. On the other hand, PMNs are much more easily obtained and multiple samples can be tested at intervals during the incubation period of illness. Consequently, while we may continue to make some observations of PAMs the bronchial immunoglobulins, the major emphasis on the future research will be on alterations in PMNs, since it seems likely that these cells will ultimately be more suitable for use in disease detection.

IV. Methods

LYMPHOCYTE SEPARATION - LAVAGE

1. Spin lavage sample 2000 rpm J-21 and collect the cell pellet i.e. resuspend cells in ≅ 5 ml PBS (~6 x 10^6/cc)

2. Add Technicon lymphocyte separating solution 4 x the volume of the cell suspension, i.e. 20 ml

   Technicon ® No. T01-0507

3. Rotate at 37°C/1 hr.
   Stand at 37°C/30 min

4. Push the cell suspension across the magnet field and collect sample

5. Spin 2000 rpm MSE, Wash cells 2-3 times with saline

6. After final wash resuspend the cells with PBS

LYMPHOCYTE SEPARATION FROM WHOLE HUMAN BLOOD

Materials:

Sodium Hypaque, 50% (w/v) "Winthrop Laboratories"
Ficoll (Mol. Wt. 400,000) "Pharmacia"
Blood drawing materials
Centrifuge with a horizontal rotor
Heparin (10,000 units/cc) or EDTA
0.9% Sodium chloride solution (or Tris buffer, Hanks' BSS, or any good buffer solution)

Procedure:

1. Solution "1". Prepare a high density solution of Hypaque and Ficoll by mixing 120 cc of sterile 9% Ficoll in water (autoclavable) and 30 cc of sterile Hypaque. This gives a solution of 6% Ficoll and 10% Hypaque with a osmolarity of 1.12 plasma equivalents. Store at 4°C.

2. Solution "2". To 100 cc of saline or physiological buffer solution add 0.1 cc Heparin (10,000 units/cc) resulting in 10 units anti-coagulant per milliliter. (EDTA can be substituted at 1 mg/ml, as the anti-coagulant)

3. Asceptically pipet 4 cc of solution "1", Ficoll-Hypaque, into a clean sterile plastic 15cc centrifuge tube (or siliconized glass tube). Set up one tube for every two milliliters of blood to be drawn.
If 8 to 28 cc of blood is required, pipet 10 cc of solution "1" into a 50 cc centrifuge tube, and set up one tube for every seven milliliters of blood to be drawn.

4. Draw the volume of blood required and mix with the heparinized buffer solution making a 1:4 dilution (1 cc blood with 3 cc buffer). Mix well to prevent coagulation.

5. Carefully pipet 8 cc of the diluted blood mixture into a 15 cc centrifuge tube containing Ficoll-Hypaque (step 3), layering the blood on top of the Ficoll-Hypaque.
Up to 28 cc of diluted blood can be layered on the 10 cc Ficoll-Hypaque solution in a 50 cc centrifuge tube.

6. Place the filled centrifuge tubes in a "swinging bucket" Rotor and centrifuge the blood for 35 to 40 minutes at room temperature with a force of 400 xg at the blood-Ficoll interface.

7. Following centrifugation the lymphocytes and monocytes will form an opaque band at the original blood-Ficoll interface, and the red cells and granulocytes will form a pellet in the bottom.

8. Using a sterile pasteur pipette, carefully remove the lymphocyte layer and transfer it into a second tube for further processing as dictated by the particular experiment.

Reference: Boyum, A., Isolation of Mononuclear Cells and Granulocytes from Human blood. Scand. J. Clin. and Lab. Invest. 21 - Suppl. 97, 77-90, 1968.

ROSETTE TEST

1. Blood lymphocytes: Separate using Ficoll-Hypaque
   Lavage lymphocytes: Separate using Technicon lymphocyte separating solution
2. Wash lymphocyte suspension 2x with PBS ph 7.2 after separation.
3. Resuspend lymphocytes with PBS and count.
4. Remove 1 x 10^6 lymphocytes and place in small round bottom tube. Spin 1700 rpm MSE - remove supernatant and resuspend cells in 0.25 ml PBS-EDTA.
5. Wash Sheep RBC's 3x with PBS. Adjust 80 x 10^6 ml with PBS
6. Test 0.25 ml containing 1 x 10^6 lymphocytes
   0.25 ml sheep RBC's in 20 x 10^6 (total)
7. Incubate 5 min/37°C.
8. Spin 200g (1100 rpm-MSE) for 5 minutes.
9. Incubate in ice bath 1-2 hours.
10. Remove portion of supernatant and gently resuspend and read.

    Positive Rosette - Any lymphocyte with more than three Sheep RBC's attached.

MICRO-TECHNIQUE

1. Prepare lymphocytes as described above. Remove 2 x 10^5 lymphocytes and place in 1 ml Bel-art plastic tube.
2. Spin 1700 rpm-MSE for 5 min. and draw off supernatant. Resuspend lymphocytes in 0.05 ml PBS.
3. Test 0.05 ml containing 2 x 10^5 lymphocytes
   0.05 ml Sheep RBC's at 80 x 10^6/ml i.e. total of 4 x 10^6
4. Incubate and read - same as macro rosette

Jondal, M., Holm, G., Wigzell, H. 1972. Surface markers on Human T & B lymphocytes. J. Exp. Med. 136:207-215.

EAC' LYMPHOCYTES

1. Wash sheep RBC 3 x in PBS
   Resuspend at 5% in PBS
2. Add equal volume of a 1:2000 dilution of amboceptor in PBS
3. Incubate at 37°C / 30 min.
4. Wash and resuspend at 5%.
5. Add equal volume of 1:20 dilution of Guinea Pig Complement
6. Incubate at 37° C / 30 min.
7. Wash 3 x and resuspend in PBS with 0.01 M EDTA
   Adjust to 100 x 10^6/ml.
8. Test 0.5 ml containing 1 x 10^6 lymphocytes PBS with 0.01 M EDTA
   0.5 ml SRBC's at 100 x 10^6/ml
9. Incubate at 37° C / 15 min.
10. Centrifuge at 200 x g/5 min.
11. Aspirate most of supernatant
12. Vigorously resuspend pellet
13. Transfer aliquot to hamacytometer and count 200 lymphocytes. Determining % of rosettes (Figure 24).

MICRO TECHNIQUE

Test 0.1 ml PBS with EDTA containing 2 x 10^5 lymphocytes
0.1 ml PBS with EDTA containing 10 x 10^6 SRBC's

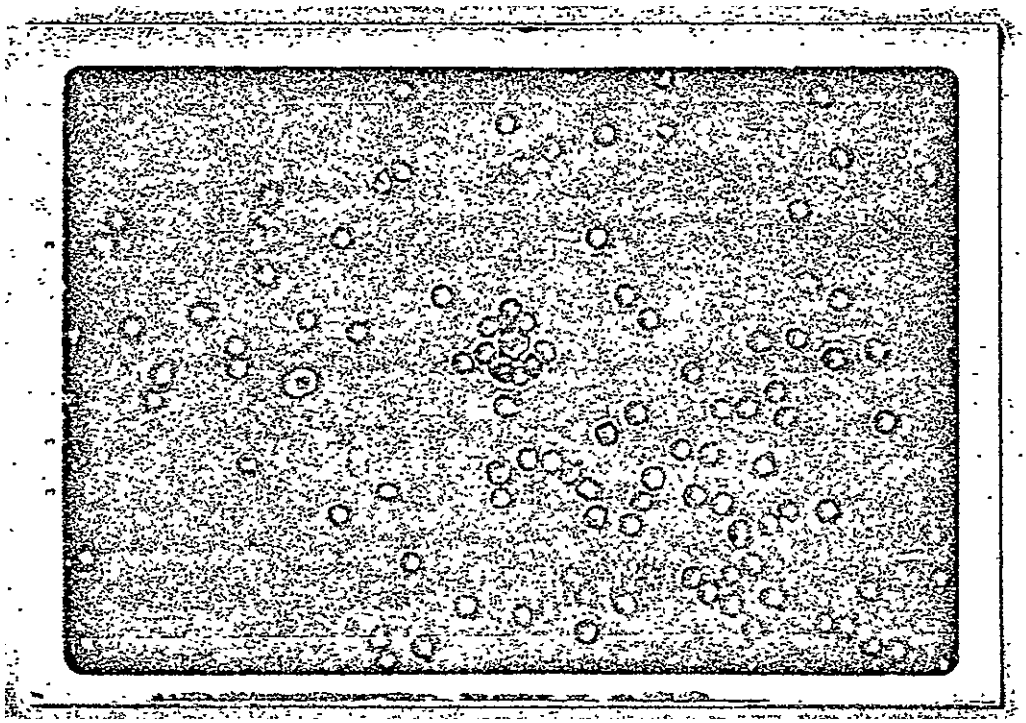
Incubation and reading same as the above

Note: Must use round bottom tube

Fernando Aiuti, et al. Surface markers on lymphocytes of patients with infectious disease. Inf. and Immunity 8:110-117, 1973.

FIGURE 24

Positive Bronchial Lymphocyte EAC' Rosette



FLUORESCENT ANTIBODY STAINING OF LYMPHOCYTES

Use lymphocytes that have been separated using Hypaque-Ficoll.

1. Spin down samples in Fisher tubes at 2500 rpm for 2 minutes (or 2000g for 3 minutes).

2. Wash each sample with cold PBS x 3.

3. On third wash divide each sample into 5 parts (0.2 ml each, marked unstained. Total anti Ig, anti IgM, anti IgG, and anti IgA).

4. Spin samples down and withdraw supernatant.

5. In each subsample add 2-3 drops of appropriate stain to pellet and mix thoroughly, but gently. Do not make bubbles. Use 1:8 dilution of stain (try 1:4, 1:8, 1:16).

6. Place samples in refrigerator for 30 minutes.

7. Top each tube with cold PBS and mix.

8. Spin cells at 2500 rpm for 2 minutes. Wash x 2 with cold PBS. Remove supernatant.

9. Using the remainder of lymphocyte sample in the bottom of Fisher tubes, take a drop and place it on a glass slide (slides used for fl. microscopy) and coverslip with a 1 1/2 coverslip. Seal with clear nail polish. Store in a dark, cool place (refrig) unitl they are ready to read.

Take another drop - add trypan blue, mix and place in a hemocytometer. Do a viability and also a diff.

IV. Problems related to Time Impact.

No major problems arose to create a major impact in our study program. The Cellular Analysis Laboratory yielded valuable support to our studies and were extremely helpful in accommodating our requests as well as our rather inflexible experiment schedule particularly where our prison volunteer studies were involved. Dr. S. Kimzey personally added valuable suggestions and significantly contributed as an informed discussant in the scientific sessions held weekly on some phase of this work. His expertise and cooperation were significant factors, we feel, in the success of this program, a fact that we wish not to go unrecognized.

V. Recommended Series of Teststo Evaluate Early Respiratory Viral Infection in Potential Astronauts.

The following tests yielded the earliest indications of infection in the diseases so far studied.

1. Viral detection by fluorescent antisera using a selected panel of specific known antibodies. (Use throat swabs and nose swabs.)
2. B and T lymphocyte absolute values in peripheral blood. The B lymphocytes significantly changed prior to clinical symptoms during flu. T cells drop with appearance of symptoms.
3. Polymorphonuclear leukocyte phagocytic index changes. This also began to drop prior to and concomitant with infection. Changes in enzyme patterns in these cells also appears to be a potential early index of infection.

C.3