

*Sorry this is  
so rough.*

## EXPERIMENTAL APPROACH

BAYLOR COLLEGE OF MEDICINE  
and  
THE METHODIST HOSPITAL

MANNED SPACECRAFT CENTER -- NASA

"CELLULAR ANALYSIS LABORATORY"

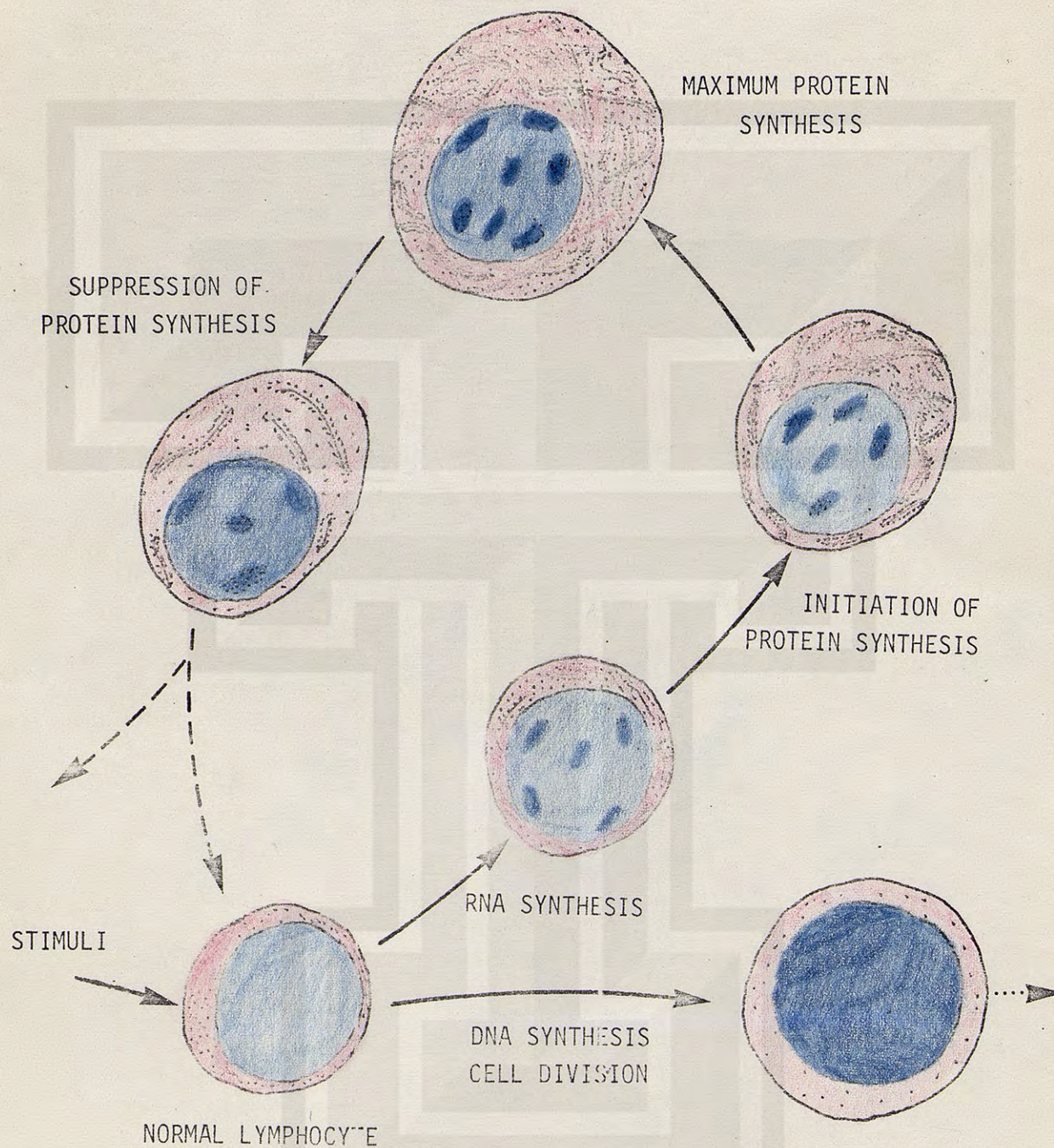
Patient Samples

- |                                                                                                                                                           |             |                                                                                                                                                                                   |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. Respiratory epithelial cells<br>on swabs or in suspension for<br>A. Conventional viral diagnosis,<br>B. Immunofluorescence, and<br>C. Radioimmunoassay | — — — — — → | 1. Cytofluorometric Analyses using<br>a Cytofluorograf-                                                                                                                           |
| 2. Leukocytes                                                                                                                                             |             |                                                                                                                                                                                   |
| A. Polymorphonuclear Leukocytes--<br>Chemotactic analysis                                                                                                 | — — — — — → | 2. Count number of responding cells<br>using Millipore Particle Counter                                                                                                           |
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| 3. Macrophages--<br>A. Chemotactic Studies and<br>B. MIF Analyses.                                                                                        | — — — — — → | 4. A. Microspectrophotometric<br>analyses using a Zeiss<br>Ultramicrospectrophotometer;<br>B. Cell sizing by use of a Millipore<br>Particle Counter.                              |



Blastogenic  
Cell  
Cycle





## LYMPHOCYTE BLASTOGENESIS



# FLUORESCENT ANTIBODY REACTION

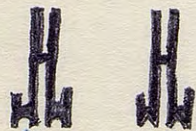
ANTIGEN

VIRUS PARTICLE  
(INFLUENZA)



ANTIBODY

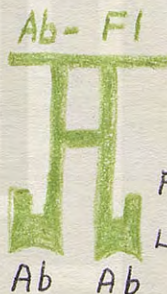
ANTI VIRAL  
ANTIBODIES



"n" shaped "v" shaped

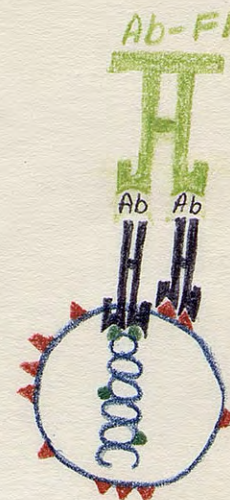


COMBINED ANTIGEN-ANTIBODY  
BUT INVISIBLE RX



FI = FLUORESCENT  
LABEL ATTACHED

ANTIBODY MADE  
AGAINST THE ANTIVIRAL  
ANTIBODIES



COMBINED ANTIGEN-  
ANTIBODY - ANTIBODY FI  
RX FLUORESCENT  
UNDER UV LIGHT

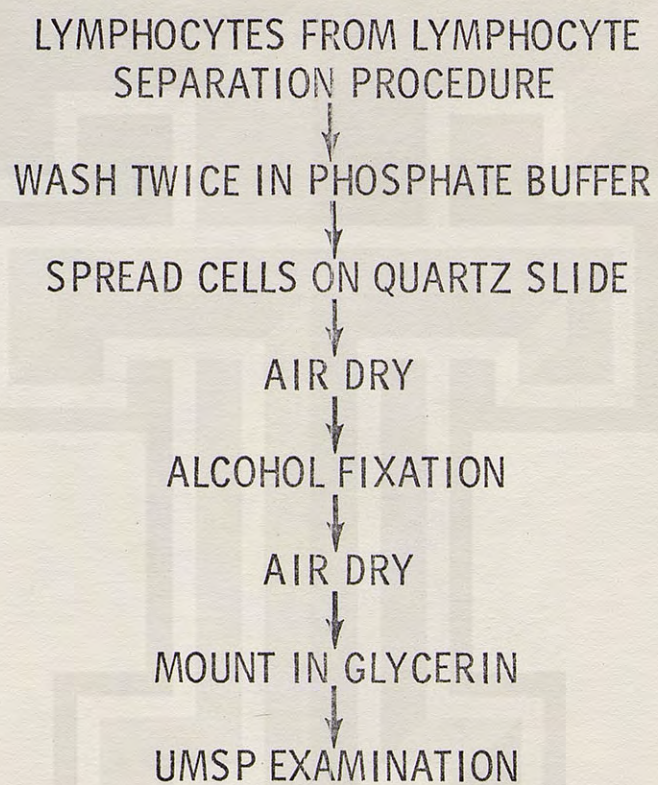


Cytophotometric  
Analyses



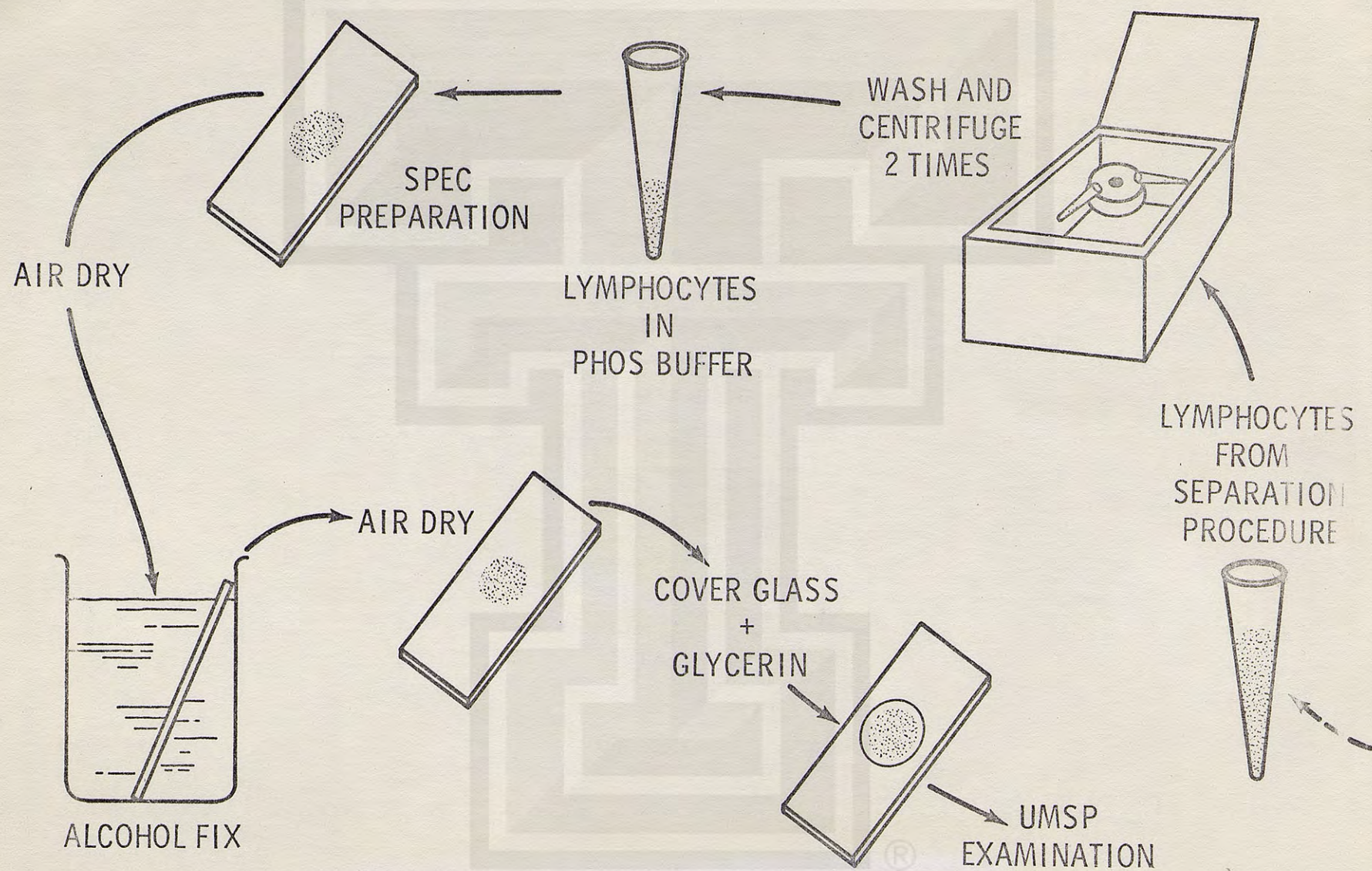


## CELL PREPARATION FOR UMSP EXAMINATION





## SPECIMEN SLIDE PREPARATION





## DATA ACQUISITION AND ANALYSIS

PERCENT TRANSMISSION OF INDIVIDUAL  
POINTS THROUGHOUT THE SPECIMEN

↓  
PHOTOELECTRIC CELL

↓  
ANALOG-TO-DIGITAL CONVERTER → HIGH SPEED PAPER TAPE PUNCH

↓  
PDP-12 COMPUTER ←

↓  
RAW DATA TAPE → RAW DATA PRINT-OUT OF CELL

↓  
REDUCED DATA TAPE

↓  
APPLICATION OF COMPUTER  
PROGRAMS FOR DATA  
ANALYSIS



PROGRESS REPORT FOR FIRST 3 MONTHS

August 1972 through October 1972

for

Contract No. NAS 9-13139

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 is to evaluate the early cellular responses of specific components of the leucocyte and epithelial cell populations to foreign challenges of both an infectious and noninfectious character. Procedures for screening potential flight crews will be developed, documented, and tested on a control population.

The first three months have been devoted to selection of systems, tests, and procedures for evaluating the lymphocyte, the viral-tissue culture, and the macrophage. A number of preliminary tests have been completed and will be described in the following report.

## II. Progress toward Overall Goal:

The achievements of the first three months are in line with the proposed time schedule submitted with the original contract. Sections 3.2.1 and 3.2.4 were to be completed in the first 3 month period, and these have been accomplished. Progress toward establishing the procedures for section 3.2.2.a, b, and c have begun one month prior to the estimated time. Antisera preparation for section 3.2.3 has begun; however, this will not be completed in the 0 to 6 month period estimated since more than 4 viruses have been selected for antisera preparation. This time period will be extended over the first year. At least 4 antisera will be prepared and purification completed during the first 6 months as we originally proposed.



### III. Progress on Sectional Phases:

#### A. Lymphocytes (3.2.1 A.)

Sample preparations: A comparative study was run on peripheral blood lymphocytes to determine the best method for separating these cells from other whole blood components. The two most commonly used methods were compared with cultural results obtained from lymphocytes separated by the Technicon Lymphocyte Separator (Technicon Instruments, N.Y.) technique.

The three procedures used were as follows:

##### 1) Glass bead column technique:

1. Type the blood sample. Pour it into a graduated centrifuge tube and add 2 ml. 5% PVP/10 ml. blood.
2. Mix by inverting tube 5X.
3. Centrifuge on setting 3/4 on ICC centrifuge for 10 min.
4. Save 1 fisher tube of serum for later.
5. Remove buffy coat and put in fisher tube.
6. Centrifuge at 2500 rpm for 2 min.
7. Remove buffy coat and place into cup (take some of the serum with it) and add antisera in equal volume.
8. Place on shaker under a light source for 5 min.
9. Let stand for 5 min.
10. Place liquid from cup in a fisher tube, leaving agglutinated RBC's behind.
11. Do a "quickie" spin in fisher tube to remove any RBC's.
12. Place supernatant in a clean tube and spin at 2500g for 2 min.
13. Resuspend the pellet in a 1:1 solution of patient's serum and Hanks.
14. Place on straw column.
15. Incubate at 37°C in waterbath for 10 min.



16. Flush straw with McCoy's medium into approx. 2-4 fisher tubes.
17. Spin tubes at 2500 rpm for 2 min.
18. Resuspend pellets (combining them in one tube) in 1 ml McCoy's.
19. Count on Hemacytometer. Use 5 small squares.

2) Ficol density separation:

Coat all glassware with Siliclad to prevent lymphocytes from sticking.

Preparation of Reagents:

1. Ficoll: 100 grams to 1100 cc. d.H<sub>2</sub>O (makes 9% soln.) Autoclave 15 min on Tiquid cycle to dissolve and sterilize. Keep refrigerated.
2. Hypaic: Dissolve 50 mls. in 83 mls. sterile H<sub>2</sub>O, rinsing bottle thoroughly. (makes a 33.29% sol'n.). Keep refrigerated.
3. Tris-Buffer (for removing RBC's from prep.):
  - a. 4.1188 grams Tris Trizma base dissolved in 200 ml. d.H<sub>2</sub>O
  - b. pH to 7.65 with HCl
  - c. 14.94 grams NH<sub>4</sub>Cl dissolved in 1800 ml d.H<sub>2</sub>O
  - d. Add Tris
  - e. pH to 7.2 with HCl
  - f. before using warm it to 37°C.

Procedure:

1. Defibrinate blood and dilute 3:1 (saline:blood) with sterile saline.
2. Layer blood over ficol (approx. 3 part blood to 1 part ficol, or as to give blood a good distance to travel down to bottom of tube).
3. Centrifuge at 500 g or 1500 rpm (check chart for Fisher centrifuge) for 40 min or 2500 for 15 min (gives more RBC contamination on higher speed and shorter time)
4. With pipette, combine Lymphocyte layers (filling tubes 1/2 full). Usually get 90% lymphocytes and 10% monocytes and polymorphs.



5. Fill tube containing lymphocytes with Tris, mix, and let sit for 10 min. (If need to remove RBC's) Spin at 1000 for 10 min. to get pellet. Tris again if RBC's are still present.
6. Rinse cells about 2X with media to remove Tris and Ficol.
7. If do not Tris, dilute the lymphocytes 1 part cell suspension to 1 part MEM so that when spun to get button cells will all spin down and the gradient will no longer be present.
8. Add 1 ml cells ( $1 \times 10^6$ ) to 2 ml MEM. After culturing should have no less than 96% viability.

Ficol - Hypaic Solution: (Make fresh each day)

1 cc Hypaic to 2.4 cc Ficol

3) Technicon technique:

If lymphocytes are to be cultured later, the blood sample should be collected in a sterile, heparinized tube and all supplies used hereafter in the separation must be sterile.

If lymphocytes are not to be cultured, non-sterile supplies can be used.

1. Remove separating reagent from refrigerator and place in a 37°C incubator or H<sub>2</sub>O bath for approx. 1 hr or allow to sit at room temp. for 2 hrs.
2. Collect blood in a 15 ml vacutainer tube containing 3 ml Na Heparin. Make these tubes up in advance to keep on hand. If another size vacutainer is used, use 4 ml Na Heparin/20 ml blood.
3. Type the blood with ABO typing serum.
4. If more than 5 mls of blood are collected, the sample may be spun down at setting 3/4 on ICC centrifuge for 15-20 min. Take off most of the serum and save. Remove the buffy coat generously, taking some RBC's and the remaining serum that was left and place in a vial.



5. Take separating reagent and shake well to mix the iron filings. Using a 50 cc B-D syringe and a 20g. needle, withdraw reagent in the amount of 4X the volume of blood.

Ex.: For 5 ml of blood, use 20 ml reagent.

6. Withdraw into the syringe approx. 1 ml of typing antisera according to the person's blood type.
7. If the buffy coat is being used, mix it well with a pasteur pipette. Draw the blood into the syringe containing the reagent and antisera.
8. Draw an airspace into syringe equal to the volume of the mixture and cap the needle with a vinyl cap.
9. Mix by inverting several times.
10. Place the syringe on Technicon rotator which is kept in a 37°C incubator. Allow to rotate for 30 min.
11. Remove syringe from rotator, remove the airspace and tap out any air bubbles present, and replace the needle with a clean one.
12. Allow syringe to sit in the rack (also in incubator) for 30 min.
13. Take the sample through the Technicon Separator. If the sample is to be kept sterile, wipe the tubing and the top of the collection tube and its holder with alcohol and allow to dry before inserting needles.
14. Spin the collected sample in the tube for 10 min at speed 3/4 on ICC centrifuge.
15. Remove the supernatant and discard.
16. Using a long pasteur pipette, extract the pellet, rinsing with MEM, into a fisher tube.
17. Spin at 2500rpm for 2 min or 1500 rpm for 3 min. Remove supernatant.
18. Rinse pellet 2X with MEM as above and place in 1 ml MEM.
19. Mix well and count on Hemacytometer. Count the 5 small squares.
20. If the sample has too many RBC's to count, use diluting pipette. Count the 4 large squares.



Cultures stimulated with phytohemagglutinin and poke weed mitogen were run by the following technique and the DNA uptake determined as follows:

All sterile supplies must be used.

1. Label culture tubes as to the sample number and the antigen to be used.
2. Dilute the lymphocyte sample to  $1.0 \times 10^6$  cells/ml with person's own serum or Fetal calf serum which has been inactivated previously at  $56^{\circ}\text{C}$  for 30 min.
3. Into each culture tube place 2 ml of Minimal Essential Media (MEM) to which pen-strep and glutamine have been added.
4. Mix the lymphocyte sample well and add 1 ml of the sample to the culture tubes, making a total of 3 ml (33% is serum).
5. Add antigens.
6. Mix well and loosen caps.
7. Place in an incubator at  $37^{\circ}\text{C}$ . Allow cultures to go 3 days for PHA and 5 days for Pokeweed Mitogen. (They may go a few days longer if necessary, for convenience).
8. Harvest (can now use non-sterile supplies) by removing the supernatant (the cells will be at bottom of tube) and add 3 ml fresh MEM.
9. Do a viability count with trypan blue if necessary.
10. Lymphocytes are now ready for  $^3\text{H}$ -thymidine procedure or whatever other test is to be performed.
11. Isolate lymphocytes.
12. To  $10^6$  lymphocytes add  $1 \mu\text{c}$  in  $0.1 \text{ cc}$  of  $^3\text{H}$ -Thymidine to each sample (culture).  $^3\text{H}$ -Thymidine has been diluted with sterile water to 1.9 curies/millimole.
13. Incubate for 2 to 3 hours at  $37^{\circ}\text{C}$ .
14. Place in ice bath to chill 10-15 min. Maintain everything cold from this point on.
15. Centrifuge (refrigerated centrifuge) at 1500-2000 for 10 min. Discard supern.



16. Add 4 cc cold saline, vortex, and centrifuge as above. Discard supern. (Can freeze samples here to save).
17. Add 4 cc 5% TCA after thawing. Place on ice for 15 min and centrifuge. Discard supernatant.
18. Add 4 cc 5% TCA again, vortex, and place on ice for 10 min. Centrifuge and discard supern. (The button will be pinkish brown.)
19. Add 4 cc methanol - to decolorize RBCs - vortex, and centrifuge. Discard supernatant. (May have to repeat for more decolorizing to reduce quenching.)
20. To the button add 0.5 cc Soluene and vortex to resuspend.
21. Place in 60° water bath for 30 min. (Can refrigerate now and store overnight).
22. Add 10 ml of scintillation fluid. (To transfer the ppt from the testtube to the counting vial, add only 5 ml of scin. fluid, transfer this to the counting vial, and then add the other 5 ml to the testtube to wash out any remaining ppt and add this to the counting vial.)
23. Cover samples for 30 min in the dark before counting.
24. Count for 50 min. (Run blank for 0.5 ml soluene and scin. fluid.)

Results from determinations using these three procedures indicate that the Technicon Separator technique was best to use (Table 1). The Ficol method would be rated second and the glass bead method last. The glass bead method is least desirable because of the low number of cells recovered (average  $5-7 \times 10^5$  lymphocytes per ml whole blood). Both other methods gave 2-3 fold increases over this method.  $^3\text{H}$ -thymidine incorporation following stimulation of lymphs with PHA and pokeweed mitogens isolated from both the



TABLE 1.  
COMPARISON OF SEPARATION TECHNIQUES

| Perimeters                                             | Technicon Method                                                                                                                                                                             | Ficol Method                                                                                                             | Glass bead Method                                               |
|--------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
| 1. Viability of unstimulated cells                     |                                                                                                                                                                                              |                                                                                                                          |                                                                 |
| a. 0 hr                                                | 90-100                                                                                                                                                                                       | 90                                                                                                                       | No data                                                         |
| b. 48 hr                                               | 95                                                                                                                                                                                           | 85                                                                                                                       | No data                                                         |
| c. 72 hr                                               | 90                                                                                                                                                                                           | 85                                                                                                                       | No data                                                         |
| d. 5-7 days                                            | 90                                                                                                                                                                                           | 75                                                                                                                       | No data                                                         |
| 2. Mean number of cells recovered                      | $1.5 \times 10^6$ / ml whole bld.                                                                                                                                                            | $1.4 \times 10^6$ / ml whole bld.                                                                                        | $5-7 \times 10^5$ / ml whole bld.                               |
| 3. Purity from other WBC's                             | 95                                                                                                                                                                                           | 65                                                                                                                       | 90                                                              |
| 4. Separation time                                     | 1 hr<br>50 min                                                                                                                                                                               | 1 hr<br>30 min                                                                                                           | 1 hr<br>30 min                                                  |
| 5. RBC/lymphocyte contamination                        | 15/100                                                                                                                                                                                       | 1/100                                                                                                                    | 5/100                                                           |
| 6. Ease of maintaining sterile preparation for culture | Sterility easiest to maintain of all separation methods. All reagents used are commercially prepared and come sterile. Handling of prep. in rinsing and culturing is the same in all methods | Sterility more difficult to maintain. Ficol has to be prepared, auto-claved, and then mixed with fresh Hypaic each time. | Sterility almost impossible to maintain because of straws used. |



Ficol and Technicon methods was equal and comparable. Although the Technicon procedure required about 20 min longer to perform it is easier to maintain sterility and cells appear to remain viable longer using this method. Purity from other white cells is also greater using the separator.

Lymphocyte Culture Response to Phytohemagglutinin and Pokeweed -- Control Samples: (3.2.2.a)

As indicated previously the lymphocyte culture procedure and the assay of DNA activity by  $H^3$ -thymidine uptake is operative. Control studies have been completed to establish normal baselines in normal adult subjects. Normal ranges for unstimulated, cultured lymphocytes are 100-10,000 cpm. Normal ranges for cultured lymphocytes stimulated with PHA are 15,000 to 100,000 or 4-500 fold increase above normal controls, and with pokeweed are 15,000 to 100,000 or 4-200 fold increase above normal controls. A 4 fold increase above control cells is the lowest range considered to be normal stimulation by use of a mitogen. This standardized test is now ready to be used in comparison with the results obtained from acridine orange staining and further reading by use of the Cytofluorograf.



Preliminary studies have begun on this determination and will be discussed in the following section.

Acridine Orange Staining of Lymphocytes: (3.2.b.)

The experiments described here were designed to determine if acridine orange could be used to stain lymphocytes repeatably for use with the Cytofluorograf and if such staining could be used as an assay for lymphocyte transformation.

First, the staining technique which was in current use was investigated to see whether, if staining were done carefully, the necessary repeatability could be achieved (Experiment 1). Lymphocytes, separated from whole human blood on the same day (Technicon procedure) were stained in a darkened room, using a 1:100 dilution of 1% acridine orange. To each 2 ml of stain were added  $5 \times 10^5$  cells, Cytofluorograf readings being made as close to 8 min afterward as possible. This procedure was repeated to give 10 replicate scans of the single batch of lymphocytes. In addition, 4 scans were made after 10 min. Histograms representing number of cells vs green fluorescence (DNA content) were generated and analyzed by computing the average position of the histogram



along the fluorescence axis  $(\bar{r})^a$ . The spread of the histogram was computed as the standard deviation of that histogram.<sup>b</sup>

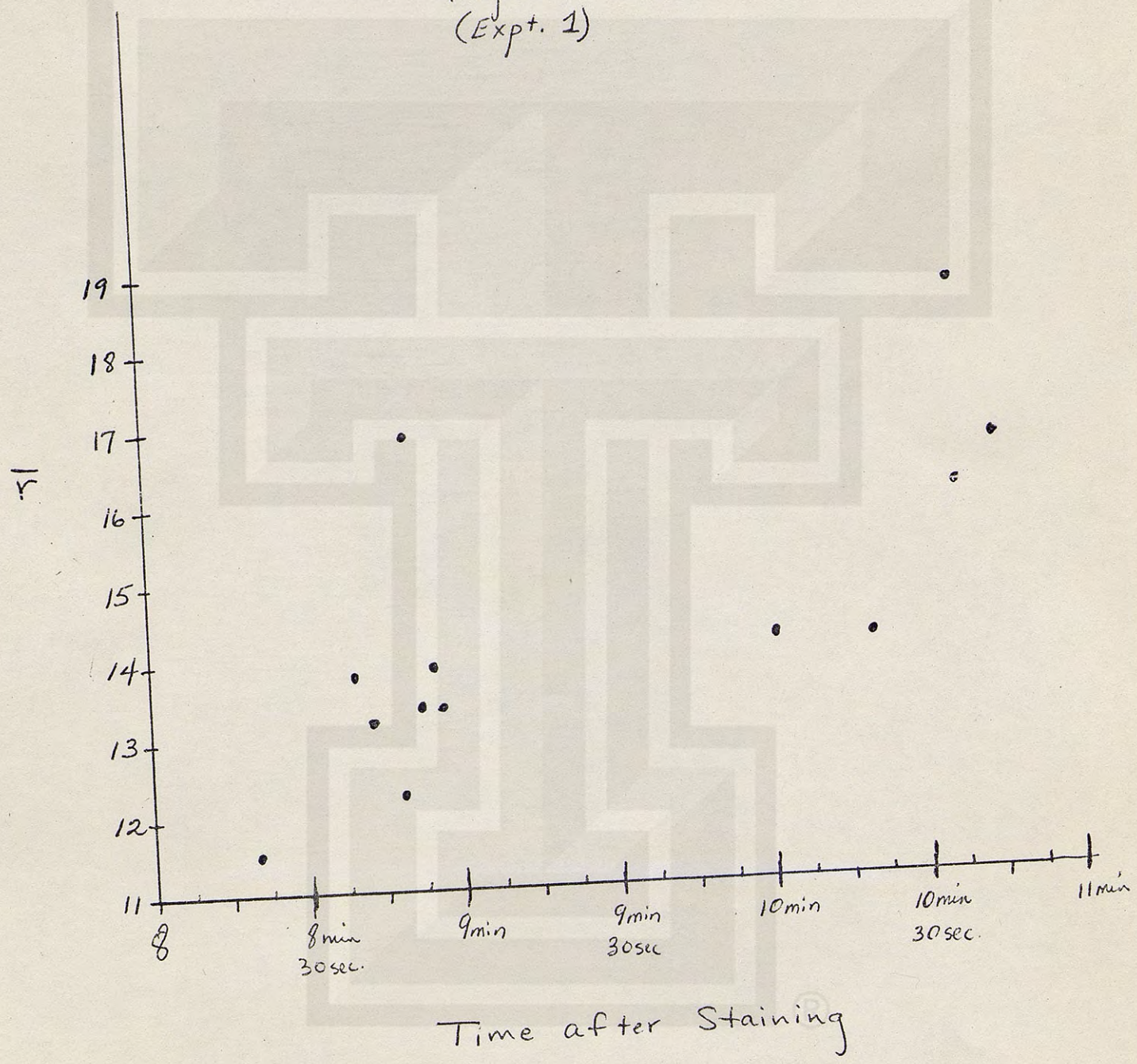
a. 
$$\bar{r} = \frac{\sum_i r_i m_i}{\sum_i m_i}$$
 where  $r_i$  is the position of the histogram bar and  $m_i$  is the height of the corresponding bar.

b. 
$$\text{s.d.} = \sqrt{\frac{\sum_i (r_i - \bar{r})^2 m_i}{\sum_i m_i}}$$

The apparent DNA content of the cells (represented by  $\bar{r}$ ) increases with time elapsed after staining (Fig. 1). This is as expected if DNA - stain binding is not yet complete by 8-10 min. If all goes well, the generation of the histogram is complete about 45 sec. after placing the sample in the instrument, and the resulting histograms are reasonably similar. In practice, however, this consistency of timing is very difficult to achieve and makes the technique too awkward to be used routinely. As contrasted with the  $\bar{r}$  values, the



Figure 1.  
(Expt. 1)





spreads of the histograms do not seem to correlate with staining time, and the range of values is relatively small (2.9 - 3.9, omitting an anomalous measurement).

Next, staining for longer periods of time at 4°C was investigated. Between 1 to 2 hours (Experiment 3), the intensity of fluorescence still shifted upward with time (Fig. 2), though the rate of shifting was, predictably, less than that for room temperature. The spread of the histograms also seemed to increase with time (Fig. 3).

If the cells were fixed and stained overnight, the results improved (Exp. 4).  $15 \times 10^6$  cells were fixed in cold 10% formalin (phosphate buffered). Cells were transferred (without removing them from the formalin solution) to cold stain and left in the cold and dark for 18 to 23 hrs. The average position of the histogram (DNA content) was calculated for 10 runs and did not correlate with time after staining (Fig. 4). The standard deviation of these 10 averages was less than 1 histogram bar. The repeatability is still not ideal, however, since for groups of three samples (a more realistic sample number), the standard deviation has been as high as 3.1. At least the remaining



Figure 2.

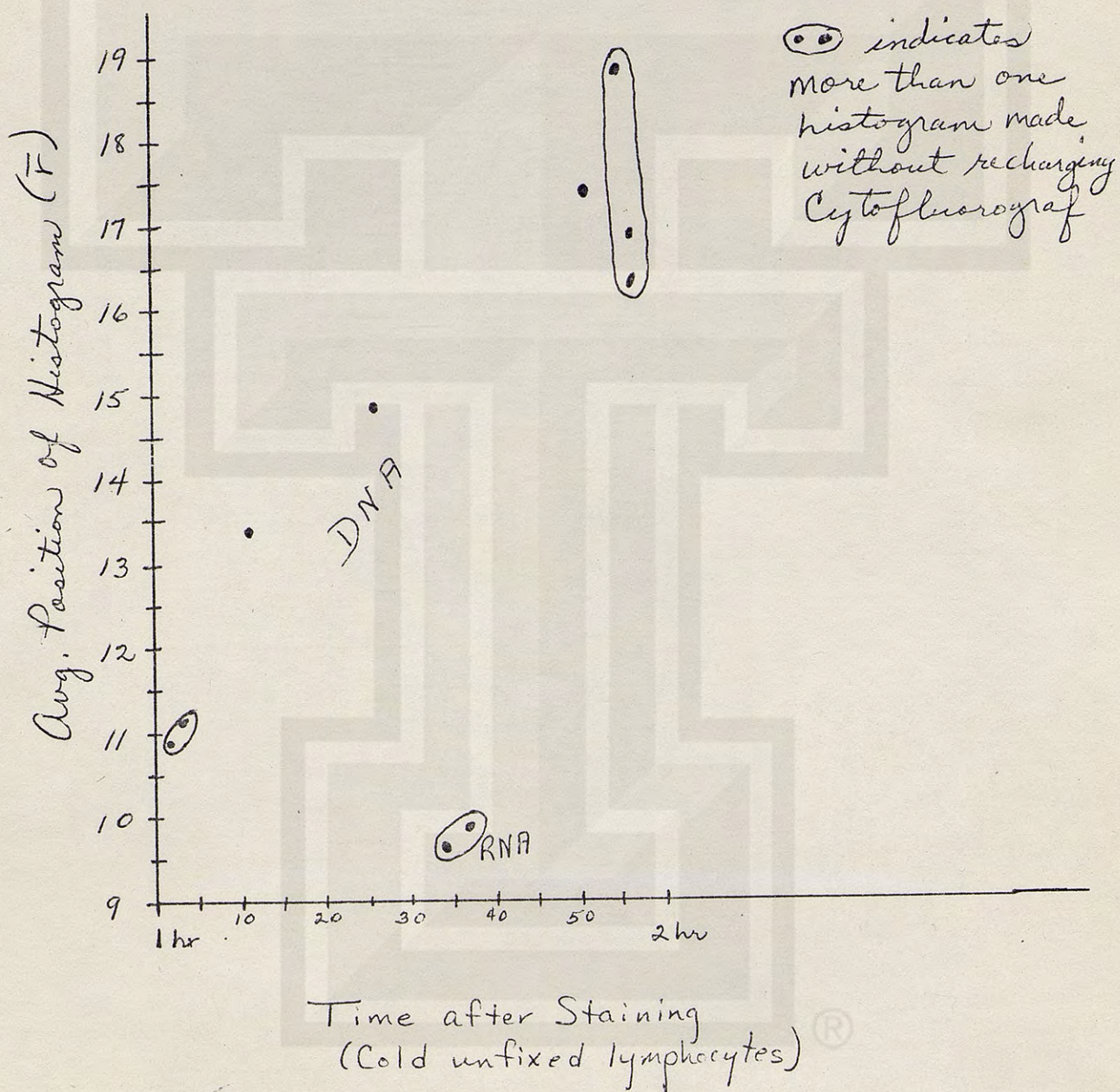




Figure 3

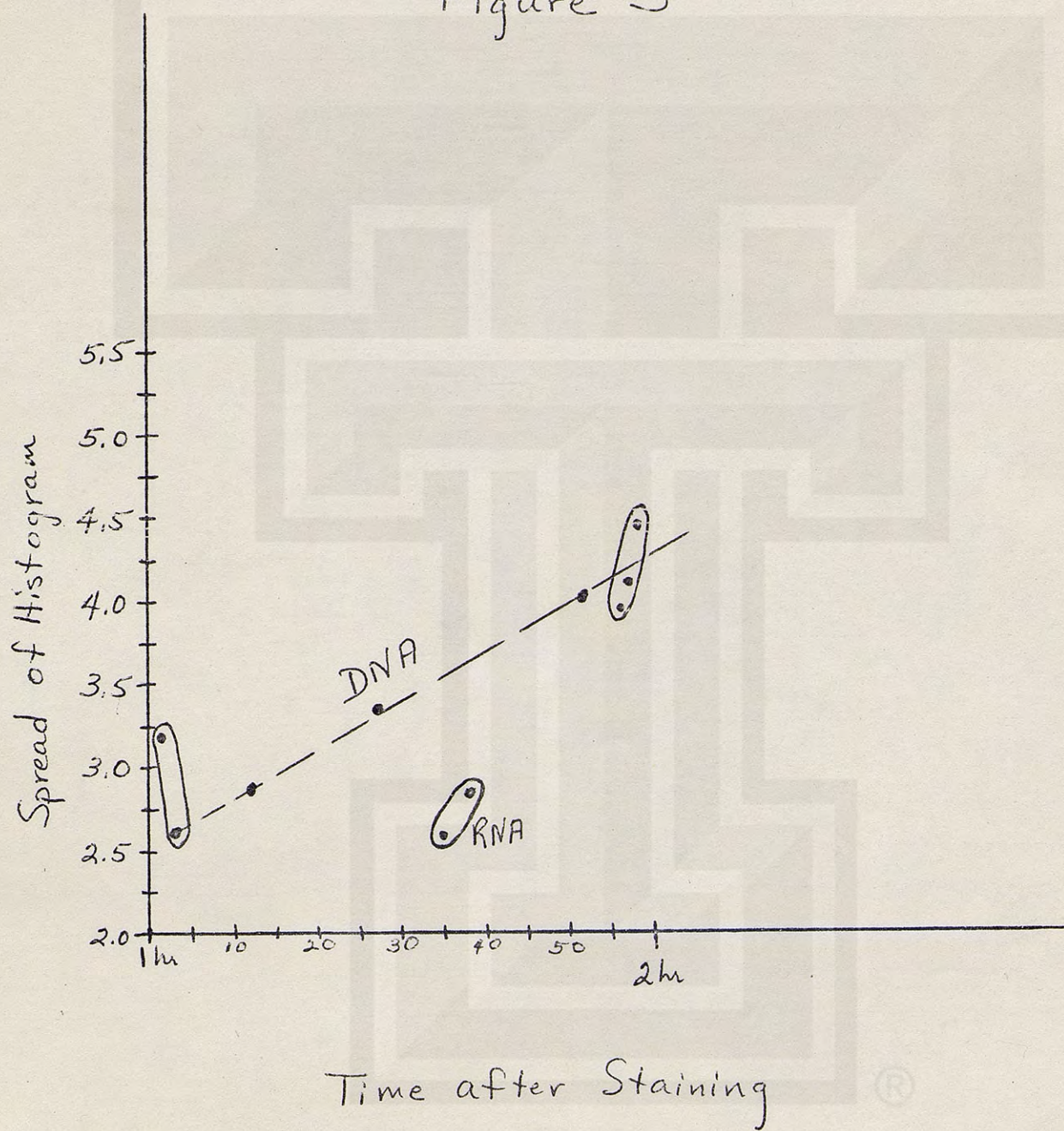
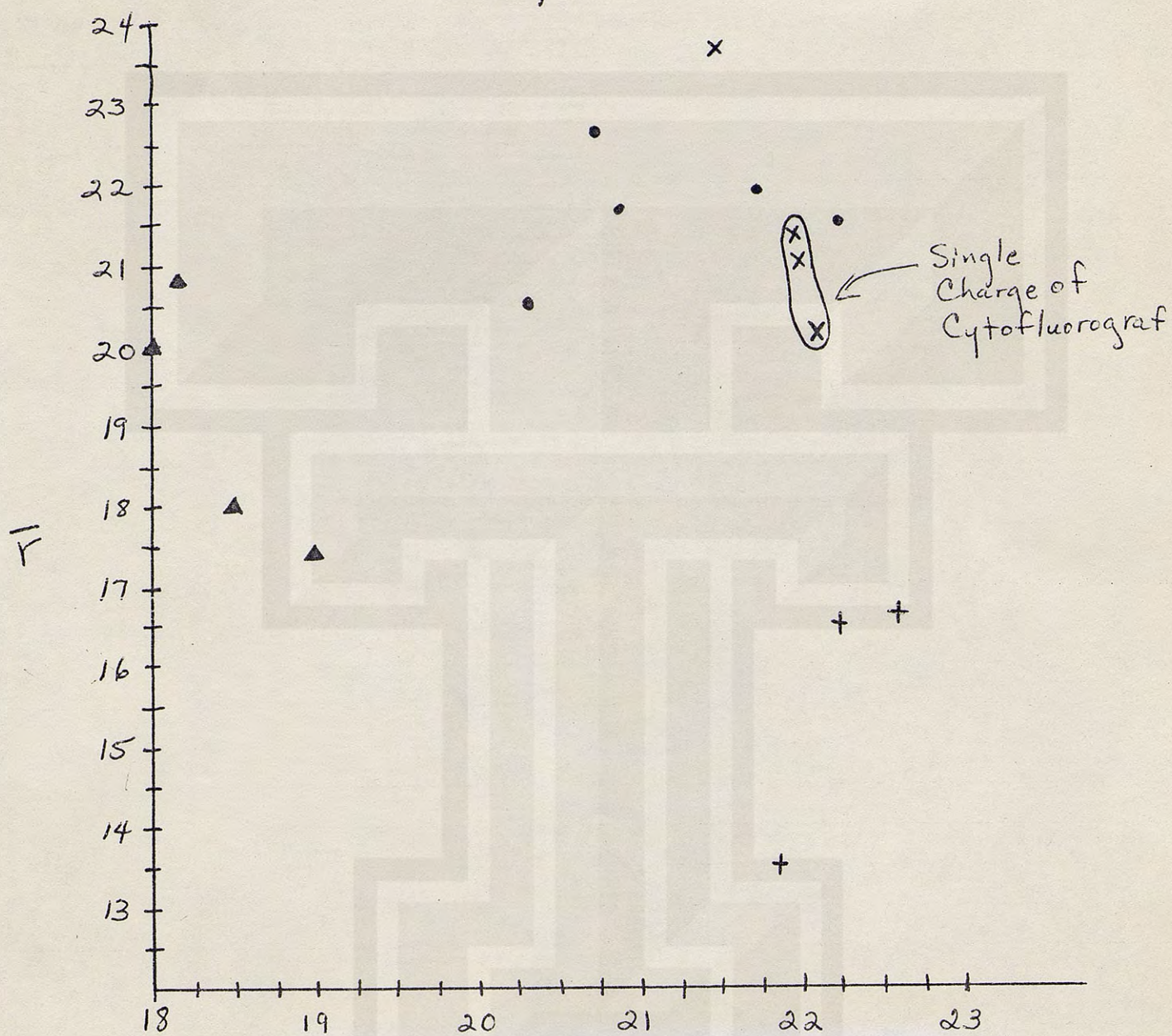




Figure 4.



Time (hr) after Staining

- + RNA - 30 min Fixation
- ▲ Fixed 3-5 hr DNA
- × Fixed 30 min. tube #2 DNA
- Fixed 30 min tube #1 DNA



variability does not reside in the staining procedure.

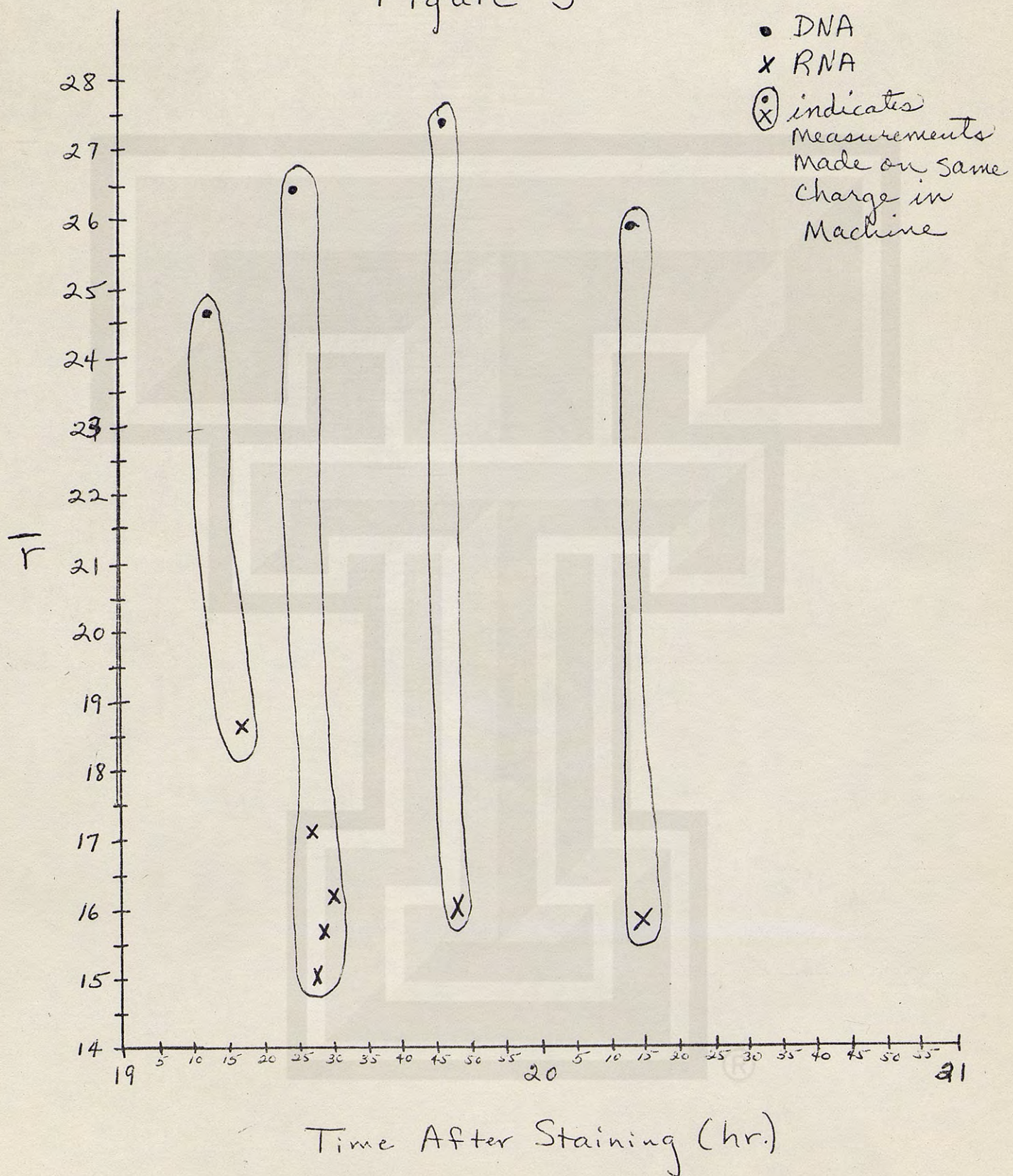
In Expt. 4, some of the cells had been fixed for 30 min and others for 3.5 hrs. The latter were characterized by a DNA histogram superficially resembling a Gaussian distribution. The histogram produced by the cells fixed for a short period has its peak in the same position as that formed by the longer-fixed cells, but there were also a small number of cells recorded in many of the higher channels, producing a ragged "tail" on the histogram curve. Similar "tails" were seen in Expt. 5 where a 2 hr 40 min fixation time was used.

If both DNA and RNA are to be assayed, the most convenient method of operating is to charge the Cytofluorograf with a sample, generate a DNA histogram, change settings, and without recharging the machine, run an RNA histogram (or vice versa), since flushing the instrument between samples is the most time consuming step. This requires that the staining be stable during the time the samples are in the instrument, about one minute. The RNA histogram seems to be stable for up to 4 min. (Expt. 5, Fig. 5), but too few measurements were made to be certain of this.

The following points should be considered before adopting this staining technique for routine use:



Figure 5





1. Does staining fixed cells in the cold give repeatable results with staining times of 1 to 4 hr? 16-18 hr? greater than 23 hr? (Note that if a 3 hr or greater fixation time is used, staining times between 4 and 16 hours are not particularly useful since they require staining or reading the samples during evening hours.) Use a constant, optimal fixation time.

2. The effects of various fixation times (e.g. 3 min to 4 hr or greater) should be investigated using a constant staining time. Might the "tail" seen with cells fixed for a short time be due to clumping? Or might the absence of this tail with longer fixation be an artifact?

3. To check the effect of the internal environment of the Cytofluorograf on stained cells, multiple histograms, both of DNA and RNA, should be made on the same sample charge.

4. Does warming the stained cells affect the results?

5. How much ambient light can the stained cells be exposed to without fading?

6. What sort of background histogram is generated using the acridine orange solution only (no cells)?



The use of the acridine orange stain as an assay for lymphocyte transformation was investigated in Expts. 2 and 6.

In the former, DNA histograms were run on three samples of lymphocytes after 5 days of stimulation with pokeweed mitogen and after 5 days of culture without the mitogen using the 8 min stain at room temperature. Difficulty was encountered in timing the runs, resulting in some of the histograms shifting to the right. However, all histograms showed a curious bimodal distribution, while with fresh, uncultured lymphocytes no more than one DNA peak was ever seen. Examination of red/green cytograms made on these samples indicated that the stimulated cultures contained a population, not present in the controls, with relatively high RNA content. Unfortunately no RNA histograms were generated.

In Expt. 6, cells from 10 individuals that had been cultured for 5 days with and without pokeweed mitogen, were fixed for 3.5 hr and stained in the cold for 17.5 to 21 hr. Visual examination of DNA and RNA histograms revealed no differences between control and stimulated cultures. Green vs. red cytograms



indicated that in most cases at least 2 populations of cells were present, differing in their DNA/RNA ratio. Whether one of these populations represents lymphocytes whose nucleic acid content has changed as a response to culturing, or represents a second cell type, such as the macrophage, growing out in culture is not known.

Again, a bimodal distribution of DNA was seen. Such a distribution is to be expected, assuming the stain as used is quantitative, for a population of dividing cells. The first peak should correspond to the normal diploid DNA content and the second to mitotic cells, with cells undergoing DNA duplication registering in the trough between the peaks. Disturbingly, the second peak seen using cultured cells lies to the left of that seen with both cultured and uncultured cells, indicating less DNA per cell than that seen in fresh lymphocytes. The nature of this peak should be investigated.

Obviously, the acridine orange method measures nucleic acid content, as opposed to synthesis as measured by isotope incorporation. This fact has 2 implications. First, it is expected that the major



effect to be seen using this technique will be a dramatic increase in the RNA content of stimulated cells, presumably detectable before DNA synthesis is measurable. On the other hand, data on DNA content would be less revealing. Unstimulated control cells, which do synthesize some DNA under culture conditions, might also show the bimodal histogram of a dividing cell population, but stimulated cells should show a greater percentage of cells in the second (doubled DNA) peak.

Second, the major diploid DNA peak should always occur in the same position for all normal human lymphocytes. Casual inspection of the data in Expt. 6 indicates that the position of the 2 DNA peaks is quite consistent (with the puzzling exception of Patient #54). The major peak is in approximately the same position in Expts. 4 and 5. Unfortunately, this peak appears on the right hand side of the histogram so that cells with twice this amount of DNA probably read off scale.

Further consideration should be given to the following:

1. Can the Cytofluorograf be adjusted to read particles of both greater and lesser fluorescence than those now being measured?



2. Can the fluorescence scale be calibrated using cells of known DNA content? e.g. sperm (assuming heterochromatin takes up acridine orange quantitatively), a polyploid human cell line, and diploid cells whose cell cycle time is known. The latter would allow quantitative correlation between a bimodal histogram and actual percentages of cells in DNA synthesis, mitosis, etc.

3. Occasionally, anomalous histograms are produced.

One cause of this seems to be clogging of the Cytofluorograf, often detectable when the histogram takes an unusually long time to generate. Since artifacts might be recognized by the absence or shift of the expected DNA peak, perhaps DNA histograms always should be run on each sample charge, even though it is RNA that is under investigation.

4. Biological variation from culture to culture should be assessed with cells from a single individual. In Expt. 6 where triplicate cultures were run, casual inspection indicates that there is little variation, but an occasional odd-looking histogram was seen. Since only one set of histograms was generated for each culture, whether this is due to biological variation is not known.



5. Measurements should be made daily on stimulated and control cells starting on Day 0. Besides allowing one to follow changes in nucleic acid content with time, effects of culturing per se to be assessed.

Determination of Surface Immunoglobulins on B Lymphocytes

(3.2.2.c.):

Preliminary studies have begun in determining the ratio of lymphocytes in the peripheral blood that have immunoglobulins on their surfaces. These cells are the B cells. The conventional method for determining the percentage of cells carrying immunoglobulin determinants is labelling the B cells with a fluorescein tagged anti-immunoglobulin antisera and manual examination of the samples by use of a fluorescent microscope. This procedure is outlined in detail as follows:

Samples of separated lymphocytes are received in a 1 ml suspension of MEM tissue culture media.

Place sample in Fisher tube and spin down at 2500 rpm for 2 min in Fisher centrifuge. Withdraw MEM supernatant. Wash sample with cold PBS (pH 7.4) three times. On third wash divide sample into 5 parts by 0.2 ml aliquots. Spin these 5 subsections at 2500 rpm for 2 min in the Fisher centrifuge. Remove supernatant. Add 2-3 drops of 1:8 dilution of fluorescein conjugated antigammaglobulin, anti-IgM, anti-IgG, and anti-IgA respectively to each pellet separately and mix thoroughly but gently. To one subsection add 1 ml of cold PBS and mix thoroughly. This



is the unstained control sample. Place samples in refrigerator for 30 min.

After the incubation period top each stained sample with PBS and mix. Spin down cells for 2 min at 2500 rpm in the Fisher centrifuge. Wash twice with cold PBS. The final cell suspension is in 1 ml of cold PBS.

Cells are now stained and ready for examination by fluorescent microscopy for use in the Cytofluorograf.

Results of this procedure are shown in Table 2 for 25 normal individuals. Literature ranges for the normal are 8-33% B cells in peripheral blood for normal individuals. This procedure is ready for a comparison study with results obtained from the Cytofluorograf.

The procedure temporarily chosen for Cytofluorograf analyses is as follows:

- 1) 0.3 ml of unstained sample is placed in the sample reservoir.
- 2) A total population count is done to insure the optimum number of cells are being run through the flow channel ( $5 \times 10^5$  to  $1 \times 10^6$ ).
- 3) 1 ml of sample is put in and at approximately 1000 cells a picture is taken with a Polaroid Tektronix camera.
- 4)  $10^5$  cells increments are then run through the flow channel to determine:
  - (a) Percent of cells falling in Grids 1-8 (Total screen).
  - (b) Percent of cells falling in Grid 1
  - (c) Percent of cells falling in Grids 2-8. This reading is taken three times.
- 5) The procedure of (4) is then repeated on the stained cells with the following exceptions:
  - (a) No total count is done on any of the stained samples.
  - (b) No picture is taken of IgM, IgA, or IgG since there is no significant difference in appearance from total stain.



Table 2

Percent B cells in Peripheral Blood of Normal Subjects

---

| <u>Sample</u> | <u>Total</u> |
|---------------|--------------|
| 1. SC(25)     | 25           |
| 2. LC(23)     | 16           |
| 3. CF(76)     | 22.6         |
| 4. JP(64)     | 15.2         |
| 5. EH(63)     | 18.3         |
| 6. NF(67)     | 13.6         |
| 7. PG(68)     | 15.7         |
| 8. MR(69)     | 12           |
| 9. WG(38)     | 10           |
| 10. LS(39)    | 6.7          |
| 11. S(77)     | 15           |
| 12. B(78)     | 5            |
| 13. S(79)     | 7.7          |
| 14. HD(80)    | 10           |
| 15. E(81)     | 9.4          |
| 16. H(82)     | 16           |
| 17. B(83)     | 16.9         |
| 18. M(84)     | 16.7         |
| 19. FD(45)    | 24           |
| 20. JTC(46)   | 22.5         |
| 21. D(85)     | 9.8          |
| 22. K(86)     | 14           |
| 23. K(87)     | 22           |
| 24. DR(88)    | 15.5         |
| 25. TG(89)    | 17.4         |



- 6) Thorough washing with 1/100 dilution Photo-Flo and water between samples is performed last.

Preliminary results indicate that the comparison of results will show fairly close correlation between the manual procedure and the Cytofluorograf.

#### B. Viral Antigen Laboratory (3.2.1.B, 3.2.3, and 3.2.4)

##### Tissue Cultures:

Tissue cultures are the most widely used system for the isolation and study of viruses. Cells, chiefly of human or monkey origin, are grown in monolayer on the wall of a test tube or other surface, and can be readily observed by microscopy. Several types of cultures are available and are generally classed as: primary, semi-continuous and continuous. Both of the former are diploid and the latter cultures are heteroploid. Primary cultures consist of cells freshly explanted from tissues, and therefore, they contain many types of cells. Examples of frequently used primary cultures are human embryonic kidney (HEK), rhesus monkey kidney (RhMK), and rabbit kidney (RK) cultures. Often such cultures may be subcultivated once giving rise to secondary cultures in which the cells are morphologically similar to those in the primary culture. Further



subcultivation without loss of the original cell types is not possible.

Certain normal tissues under optimal conditions may be subcultivated a limited number of times (about 50). Such cultures consist of fibroblast cells and are often called diploid cultures because they maintain their characteristic diploid chromosomal configuration throughout their passage history. Examples of diploid cultures are the WI-26 and WI-38 lines of human embryonic lung fibroblasts.

Heterploid cell lines can be subcultivated indefinitely. They are derived from human or animal cancers. Examples of such cell lines are Hela, Hep-2, and KB cultures.

#### Recognition of Viral Replication in Tissue Cultures:

##### 1) Cytopathic Effect (CPE):

Cytopathic effect results from the multiplication of viruses in tissue culture cells. Examples where this is the end point are picornaviruses in WI-38 cells, adenoviruses in HEK cells, and herpes simplex in rabbit kidney cells.

##### 2) Hemadsorption (HAd):

The adsorption of erythrocytes to infected cells as a result



of fixation of red blood cells to viral hemagglutinins at the cell surface is the principle method of detection of influenza and parainfluenza viruses in rhesus monkey tissue cultures. Such cultures usually do not exhibit CPE.

3) Interference:

Interference with replication and cytopathic effect of a second virus by a noncytopathogenic virus is the method used for detection of rubella virus in African green monkey kidney cultures. The indicator virus is usually echovirus type 11.

4) Immunofluorescence:

Tissue culture cells are reacted with antisera which contain known viral antigen specificities. A fluorescein tagged anti-immunoglobulin antiserum is then reacted with the tissue culture cells. If the antigens of the specific viruses are present the cells fluoresce. A number of viral systems may be studied in this manner including Influenza A, B, RSV, etc. Specific anti-viral antiserum is the principle requirement.



Most Frequently Used Cultures For Diagnostic Virology Laboratory:

| <u>Culture</u> | <u>Viruses Which May Be Isolated<br/>In Indicated Culture*</u>                                  | <u>Observation<br/>Period</u> |
|----------------|-------------------------------------------------------------------------------------------------|-------------------------------|
| WI-38          | Rhinoviruses<br>Other picornaviruses<br>Herpes Simplex<br>Adenoviruses                          | 14 days                       |
| RhMK           | Myxoviruses<br>Paraviruses<br>(except RS)<br>Coxsackie B viruses<br>Echoviruses<br>Adenoviruses | 10 days                       |
| HEK            | Adenoviruses<br>Herpes Simplex<br>Echoviruses<br>Respiratory Syncytial                          | 30 days                       |
| RK             | Herpes Simplex                                                                                  | 10 days                       |

\* System is optimal for virus at the top of each list.

Steps for Processing Clinical Specimens:

| <u>Specimens</u>                   | <u>Tissue Cultures</u> |             |            |           |
|------------------------------------|------------------------|-------------|------------|-----------|
|                                    | <u>WI-38</u>           | <u>RhMK</u> | <u>HEK</u> | <u>RK</u> |
| Throat or oral swab                | X                      | X           | X          | X         |
| Anal Swab                          | X                      | X           | X          |           |
| Nasal swab or<br>nasal-throat swab | X                      | X           | X          | X         |
| Lesion swab or scraping            | X                      |             |            | X         |
| Sputum                             | X                      | X           | X          | X         |
| CSF                                | X                      | X           |            | X         |



| <u>Specimens</u>  | <u>Tissue Cultures</u> |             |            |           |
|-------------------|------------------------|-------------|------------|-----------|
|                   | <u>WI-38</u>           | <u>RhMK</u> | <u>HEK</u> | <u>RK</u> |
| Urine             | X                      | X           |            | X         |
| Heparinized Blood | X                      | X           | X          | X         |
| Clotted Blood     | X                      | X           | X          | X         |
| Conjunctival swab | X                      |             | X          | X         |
| Autopsy Specimen  | X                      | X           | X          | X         |
| Biopsy Specimen   | X                      | X           | X          | X         |
| Other             | X                      | X           | X          | X         |

Hep-2-tubes can be substituted for HEK tubes when HEK's are not available.

Virus Isolation From Specimens:

1. Within 24 hours prior to inoculation change media on tissue culture cells.
2. Inoculate 0.4 ml of specimen per tissue culture tube, down the side of the tube opposite the company insignia (location of cell layer). Keep two uninoculated tubes per cell line as controls to be used in daily readings.
3. Incubate on a roller drum at 34°C for a minimum of 2 hours and a maximum of 24 hours.
4. Decant media and re-feed with 1.5 ml of media (for WI-38 cell line use MEM with 2% calf serum + antibiotics).
5. Return tubes to roller drum, incubating at 34°C.



Read daily for cytopathic effect (CPE), recording observations.

CPE Gratings:

- 0 = negative
- <5 = less than 5 foci/cell sheet
- 0-5 = > 5 foci but less than 1+
- 1+ = approximately 1 focus/microscopic field
- 2+ = 25 to 50% of cell sheet involved
- 3+ = 50 to 75% of cell sheet involved
- 4+ = 75 to 100% of cell sheet involved

6. Harvest (freeze at  $-70^{\circ}\text{C}$ ) tissue culture tubes when CPE reaches 4+ reading. You will use the positive tubes later when you run a neutralization identity. If tubes are negative, maintain them for indicated period for appropriate virus before discarding. To maintain a good cell sheet, change the media on the tissue approximately every third or fourth day or when the fluid in the tubes appears acid (yellow). Never allow fluid to turn to a lemon yellow because cells will become toxic and die. Do not change the media on a tube without reading for CPE first. Do not harvest a positive tube if fresh media has been added to the tissue culture tube. Allow 24 hours for virus replication, then harvest the tube.



Hemadsorption (HAd) of Monkey Kidney Tissue:

1. Label vial (1 dram) same as tissue culture tube. Add 0.03 ml 10% gelatin.
2. Decant media from tissue culture tube into vial and refrigerate.
3. Wash tissue culture tube four times with 1 ml of 0.1% guinea pig red blood cells in sterile saline leaving the last ml in the tube.
4. Refrigerate at 4°C for 20 minutes with red blood cells on the tissue.
5. Read and record results.
6. Wash tissue culture tubes 3 times with 1 ml of Hanks BSS (Balanced Salt Solution) with Penicillin, Streptomycin, and Amphotericin. Re-feed with 1.5 ml media and return to roller drum.

Samples Processed for August and September:

1. August

93 clinical specimens received  
31 reports sent out:  
24 negative  
4 Enteroviruses  
1 Herpes Zoster  
1 CMV  
1 Rhinovirus



## 2. September

59 Clinical specimens received:

64 reports sent out:

48 negative

4 Enterovirus

1 Herpes Zoster

1 Herpes Simplex

6 CMV

### C. Macrophage Studies

#### Studies of the Effect of Cigarette Smoking on Pulmonary Alveolar

##### Macrophage:

Pulmonary alveolar macrophages were obtained by saline lavage from 4 non-smokers and from 4 cigarette smokers. These have been studied using the Zeiss Microspectrophotometer to scan individual cells at several different wavelengths. These studies are being done in an attempt to define characteristics of the normal macrophage, so that early alterations in the macrophages due to disease or other pathological conditions may be detected.

Familiarization with the Zeiss Microspectrophotometer: (Preparation of macrophage smears on quartz slides)

1. Clean quartz slide with absolute alcohol, rinse thoroughly with distilled water and air dry. With a lint-free cloth or lens paper, remove all lint from slide surfaces.



2. Place drop of macrophage suspension on quartz slide and incubate thirty minutes in a moist chamber at  $37^{\circ}\text{C}$ . Remove slide from chamber and air dry rapidly.

3. Place dried smear in absolute alcohol for 15 minutes. Rinse with distilled water and allow to air dry.

4. Fixed smears can be stored in a lint free container until needed.

5. Cover-slip smears for observation. Place a drop of immersion glycerin on smear and cover with a rectangular quartz cover-slip. Gently press the cover-slip down on smear to remove excess glycerin. Seal edges on the cover-slip with clear finger nail polish.

#### Morphological Classification of Pulmonary Macrophages:

1. Macrophages were classified on the basis of their morphology under the light microscope into four different types. These range from smaller cells with no cytoplasmic inclusion material up through larger cells with abundant cytoplasmic inclusions heavy enough to obscure the detail of the nuclei.

2. Macrophages representing each of the morphological types are being scanned with the Zeiss Microspectrophotometer. Each



cell selected is being scanned at three different wave lengths (260, 280, and 310 nano-meters). Information is being stored on magnetic tape, grouping each of the morphologic cell types and each wave length scan together. After a suitable number of cells have been scanned, the stored data will be retrieved and characteristics of the different calculations of macrophages will be examined for possible significant differences.

Scanning Electron Microscopy of Macrophages from Smokers and Non-Smokers:

Macrophages from smokers and non-smokers are washed in saline and immediately fixed in glutaraldehyde in preparation for scanning electron microscopy. The surface morphology of these cells will be systematically examined.

Pulmonary Lavage of Subjects with Acute Viral Infection:

An order has been placed for an Olympus Flexible Fiberoptic Bronchoscope. When this instrument arrives, we will be able to expand our studies to include volunteers with experimental viral infection. Approval has been obtained from the Human Experimentation Committee at Baylor to study three volunteers with acute Rhinovirus infection. These volunteers will be studied before, during, and after



experimental illness. If no complications are encountered, these studies will be increased to include larger numbers of volunteers and a wider range of infecting organisms.

#### IV. Problems Related to Time Impact:

Currently, there are two areas that are presenting a time impact problem for the lymphocyte studies and the viral antigen laboratory. One is the delivery of a fluorescent attachment for the Leitz Microscopy System for use by the antigen laboratory. A second problem that is highly significant is access and personnel to calibrate and use the Cytofluorograf with the new system for data analyses. This problem could mean total loss of data on human volunteers for the acridine orange staining procedure for DNA and RNA ratio determinations. Human volunteer studies must proceed whether the instrument support is available or not. The B/T ratio study will be effected also, but these determinations will proceed with manual determinations being made. To alleviate this second problem, a technician must be hired to run this instrument. Preliminary studies reported in this study report were performed by personnel on other grants or by



students. These people are no longer available for this work.

Because of this a letter is enclosed with this report to request an amendment to the existing contract to cover costs for this person.

The macrophage studies may receive impact problems from the inoperative capacity of the Millipore particle counter unless this instrument becomes operative within a month to six weeks.



V. Anticipated Time Table:

The next three months should progress as indicated previously on the time table. The table is again included with the additional areas of new time indicated by dotted lines. (Table 3).



RESPONSE TO REQUEST FOR PROPOSAL  
NO. 9-BB 32-79-2-375P

Submitted by

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## **Volume I. Technical Proposal for 9-BB32-79-2-375P**

- I. Statement of Work**
- II. Methods and Procedures**
  - A. Sections 3.2.1 and 3.2.2a-c**
  - B. Sections 3.2.3 and 3.2.4**
  - C. Sections 3.2.5a-e, 3.2.6, and 3.2.7**
- III. Background Work Performed and References**
- IV. Names and Resumes of Personnel**
- V. Facilities Statement**
- VI. Time Schedule**

## **Volume II. Budget**

- A. Category Requirements**
- B. Summary on Form DD-633-4**



I. Statement of Work:

The statement of work that originated with the contract request is acceptable in its present form as stated and is, therefore, attached.



## Statement of Work

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

#### 1.0 General Scope

##### 1.1 Introduction

Accurate evaluation of the health status of astronaut crews prior to space flight has been a prime concern of the NASA throughout the Manned Space Flight Programs. Longer duration missions, beginning with the Skylab Program, impose new demands for high resolution assessment of the health status of crews prior to their commitment to extended flights. Routine analytical procedures currently in use in the clinical laboratory do not detect infectious processes during the incubation period. More sophisticated techniques are needed to establish screening procedures for the early detection and diagnosis of illnesses in their preclinical, asymptomatic stages. Among the earliest indications of physiological abnormalities are the responses of the cellular immune system.

##### 1.2 Objective

The objective of this effort is to evaluate the early cellular responses of specific components of the leucocyte and epithelial cell populations to foreign challenges of both an infectious and noninfectious character. Procedures for screening potential flight crews will be developed, documented, and tested on a control population.

##### 1.3 End Product

The end products of this contractual effort are to be:

- a. Documented results of laboratory experiments to determine the functional and structural alterations of leucocyte populations in response to specific and nonspecific stimuli.
- b. Defined profile of laboratory tests for screening individuals for early signs of infectious disease.
- c. Statistical analysis of the functional states of lymphocytes and macrophages in healthy and diseased individuals.
- d. A final report which documents laboratory procedures, results and analyses, and which makes specific recommendations for implementing an Early Detection of Disease Program for screening potential flight crews.

#### 2.0 Background

The ability of man to respond to foreign challenge whether viral, bacterial or protozoan depends upon the status of his immune system. The lymphocyte is



the primary cell of the immune system that is responsible for responding to a foreign challenge in such a way as to either make antibody that will help destroy the invader or transmit information to other cells that can in turn act against the invasion. The lymphocyte, like other cells, is composed of the cellular organelles including a nucleus of DNA with nucleolar bodies of RNA, mitochondria, ribosomes, and numerous membraneous channels. The lymphocyte cell membrane is arranged in such a manner as to house receptor sites for various immunoglobulins (i.e. IgG, IgM, IgA, etc.). Depending upon the origin, thymic (T) or bone marrow (B) derived, the lymphocyte appears to have either large concentrations of immunoglobulin (B cells) on their surfaces or very small and better hidden quantities in its cellular surfaces (T cells).

When the lymphocyte responds to a foreign challenge by an infectious agent a number of measurable changes can occur. The DNA-RNA concentrations and ratios change both through new synthesis and more rapid turnover rates. These changes represent some of the earliest responses in a blastogenic response of a lymphocyte. Not all the lymphocytes of the body appear to respond at one time to a particular infectious agent. For this reason, populations of lymphocytes must be studied and population shifts or changes may indicate the immune responsiveness to a disease. In some diseases states the ratio of T cells to B cells may change as well as the types of immunoglobulins (IgM, IgG, or IgA) on the surface of the B cells.

While the population of lymphocytes are changing because of the entry of such an agent as a virus into the body, other cells such as epithelial cells which line the respiratory cavity may change because of viral growth within the cells. Within these cells early signs of viral development may be represented by antigen development and cytopathic effects (CPE) due to further proliferation of the viruses.

Since the macrophage is one of the first cells to come in contact with an infectious microbe entering the body, related changes in its composition could indicate very early stages of an ensuing disease. The present study will also examine macrophage function and structure. A number of different studies employing a variety of techniques have investigated the role of the macrophage in host defense against foreign substances, infectious agents, and neoplastic transformation. Most of the research has involved the use of laboratory animals. The hypotheses formulated from animal studies must be tested using human cells before conclusions can be drawn about the role of the macrophage in human disease.

Techniques are now available which allow procurement of relatively pure populations of macrophages from human volunteers. For the first time, it is feasible to embark on systematic investigations of cellular function, using viable macrophages in vitro to evaluate several properties ascribed to these cells and making correlations with morphological and biochemical observations.

### 3.0 Technical Requirements

#### 3.1 General

The contractor shall provide all personnel, equipment, supplies, and facilities necessary to complete the contractual effort as stated, with the



exception of specialized facilities of the Cellular Analytical Laboratory at MSC which will be utilized in support of this effort.

### 3.2 Specific Tasks

3.2.1 The contractor shall establish and evaluate methods for preparation of suitable populations of the various cell types (lymphocyte, macrophage, epithelial) required to perform this task.

3.2.2 The functional capacity of lymphocytes to respond to viral infections will be evaluated with respect to:

a. Blastogenic response to specific stimuli in vivo during a viral infection and to non-specific stimulation by PHA, Streptolysin-O, and pokeweed mitogen in vitro.

b. Population shifts in cellular DNA-RNA concentrations and turnover rates.

c. Immunoglobulin distribution on B cells following in vivo infections.

3.2.3 Epithelial cells will be examined for the quantity and type of associated viral antigen. The time course of antigen appearance following an infection will be established and correlated with the time course of the lymphocyte response. This examination will require preparation of specific anti-viral antisera.

3.2.4 Procedures for virus detection and isolation in cell lines will be established.

3.2.5 Macrophages lavaged from healthy volunteers will be evaluated for their normal characteristics by:

a. Their capability for the phagocytosis and killing of bacteria.

b. Responsiveness to chemotactic stimuli.

c. The effects of lymphocyte migration-inhibition factor.

d. Microspectrophotometric analysis of single cells for size, DNA/RNA ratios, and cytoplasmic inclusion material.

e. Electron and light microscopy.

3.2.6 Macrophages obtained from otherwise normal and healthy subjects who are regular cigarette smokers of varying duration and daily consumption will be studied using all the techniques outlined in Section 3.2.5.

3.2.7 Macrophages will be serially obtained from normal volunteers who will be experimentally infected with respiratory viruses, such as rhinovirus or Coxsackie A-21. Lavages will be obtained before infection, at the



height of illness, and during convalescence from infection. The in vitro functional and morphological studies listed in Section 3.2.5 will be performed to evaluate the influences of viral infection on the pulmonary macrophage.

#### 4.0 Reporting

##### 4.1 Data Analysis

Contractor is responsible for all interpretation and statistical analysis of data generated in support of this effort. Laboratory results should be made available to the Technical Monitor for review and discussion on a regular basis.

##### 4.2 Interim Report

The contractor shall prepare a quarterly report assessing progress to date. This report should include any problems which could impact the overall contract effort. Three copies of this report shall be sent to the NASA Technical Monitor and one copy to the Contracting Officer.

##### 4.3 Final Report

The contractor shall prepare a yearly final report summarizing all work done on this contract including documentation of detailed procedures, results, and analyses. It should also include recommendations for implementing an Early Detection of Disease Program for evaluating the health status of potential space flight crews. Twenty-five copies of the final report shall be sent to the NASA Technical Monitor and one copy to the Contracting Officer.

#### 5.0 Period of Performance

The Period of Performance of this contract is two years.



## II. Methods and Procedures

### A. Sections 3.2.1 and 3.2.2a-c.

Outlined under 3.0 Technical Requirements:

Blood and throat swabs will be obtained from patients with acute respiratory illness who are admitted or seen at Methodist Hospital Clinic. The physician or, upon the physician's recommendations, a qualified technical assistant will collect the samples. The work to be done will serve in a diagnostic capacity to assist the physicians in evaluating the patients. The lymphocyte determination may be done on as little as 5 cc of blood, making the test applicable even to newborn and small infants. Neither procedure should cause undue discomfort or have lasting side effects for the patients.

Lymphocytes will be separated from the blood following collection by either a glass bead column method of Terasaki (1), by a micro Ficoll technique of Noble et al (2), or by the use of the Technicon Lymphocyte Separator. Currently, these techniques are being evaluated to determine the one that yields the greatest percentage of pure lymphocytes from the least amount of blood.

Lymphocytes will be studied as follows:

(1) Blastogenic response. The response of the lymphocytes to in vivo stimulation by a viral infection will be studied by incubating  $10^6$  lymphocytes for 3 hours with  $^3\text{H}$ -thymidine and then precipitating the incorporated label and counting the uptake. The results will be compared to a baseline of normal individuals that has already been established.

Since the in vivo response may be very low, the lymphocytes will also be cultured in tissue culture. These cells will be stimulated with PHA, streptolysin-O, and poke weed mitogen. At 3-5 days  $\text{H}^3$ -thymidine will be added to the cultures, the cells incubated for 3 hours, and then the radioactive incorporation determined by harvesting and counting in a Packard liquid scintillation counter. The results will be compared to previously established baselines.

(2) DNA-RNA by cytophotometry: Separated lymphocytes will be incubated with acridine orange for 8 minutes and then run through a cytofluorograf (Biophysics, Inc.) to determine the pattern of DNA-RNA content in the population of cells. The pattern obtained will be compared to normal baseline values previously established. Slight shifts in either DNA or RNA content will be easily detectable with the use of this instrument. This method will be compared with the radioactive procedure previously described for sensitivity as well as time of detectable changes.



(3) Immunoglobulin distribution on B cells. T cells are thymic derived cells that appear to be distinguishable from B cells in that they do not stain with immunofluorescent techniques. Normal individuals have approximately 10-30% B cells in circulation with the majority of lymphocytes being T cells. In an infection, the B cell is responsible for ultimate antibody formation (3). The distribution of specific immunoglobulins of B cells during infection has not been determined.

A suspension of lymphocytes separated from whole blood will be mixed with fluorescein tagged anti-gamma globulin, Anti-IgA, Anti-IgM, or Anti-IgG antisera following the method of Pernis et al (4). After 30 min incubation, the cells will be spun down, washed 3X with PBS, and placed on a hemacytometer for counting. The number of cells staining for each immunoglobulin will be determined by use of a Leitz photomicroscope equipped with an HB0200 mercury burner and compared with results obtained when the same stained cells are analyzed by cytofluorograf (Biophysics, Inc.).

#### B. Sections 3.2.3 and 3.2.4

Epithelial cells from the nasopharyngeal region obtained by swabbing will be studied to determine the time of appearance of early viral antigens in the cells. These antigens will be studied as follows:

(1) Detection by immunofluorescence using specific antisera. The viral infections to be studied will include respiratory syncytial virus (RSV), para influenza, influenza virus, and herpes.

Antiviral (specific virus) antisera will be prepared by inoculating rabbits with tissue culture extracts or appropriate commercial vaccines of each individual virus. After the initial studies of the antisera, the spectrum of viruses will be expanded to as many as 10. The rabbits will receive intramuscular injections every 2 weeks for 5 injections. One month following the last injection the animals will be bled. At monthly intervals during inoculation the animals will be bled and the neutralizing antibody determined.

The above antisera will be applied to slides onto which the throat swabs have been rolled and epithelial cells deposited. The antisera will be incubated for 30 minutes in moist chambers, washed 3 times in PBS, and then exposed to fluorescein tagged anti-rabbit gamma globulin for 30 min in a moisture chamber. After counter-staining, the slides will be washed 3X in PBS, mounted in buffered glycerol and examined under a Leitz aristophot microscope with an HB0200 mercury burner and an MPV photometer to determine amount of fluorescence. This unit may be used to give a quantitative determination of the amount of fluorescent antibody present. A number of workers (5, 6, 7, 8, 9) have found the above approach useful in detecting and screening viral diseases.



(2) Isolation and Identification: Cultures for virus isolation will be set up by routine tissue culture procedures. These will be examined for the presence of viruses at frequent intervals using the fluorescent antisera described previously (6). Other methods of isolation will include CPE determinations. Identification will be made by neutralization techniques and electron microscopy when applicable to determine the numbers of infectious particles present.

#### C. Sections 3.2.5a-e, 3.2.6, and 3.2.7

Common to all the studies is the procedure of pulmonary saline lavage for the collection of macrophages. This procedure is outlined first.

Human material will be obtained from healthy adult volunteers. The procedure will be explained in detail; and the possibility of a benign, transient post-lavage pulmonary infiltrate (which, in the absence of obstructive lung disease, is invariably gone by 24 hours) will be pointed out. The informed consent of all subjects will be a prerequisite for participation in the studies. Approximately equal numbers of nonsmokers and habitual smokers will be utilized, to allow meaningful comparison of the macrophages obtained.

The techniques of bronchial pulmonary lavage will be similar to those employed by Finley et al (10). The upper airway is anaesthetized by spraying a local anesthetic. A 19F Metras bronchographic catheter is inserted through the mouth into the bronchial tree until the tip is at the segmented bronchus level. The balloon cuff is inflated and from 100 to 200 ml of saline is lavaged. The recovered material (usually around 100 to 150 ml) is centrifuged in the cold at 150 g for 10 minutes. The cellular pellet is pooled for further washes and studies as indicated. The saline supernatant is decanted and stored at  $-70^{\circ}\text{C}$  for future studies of immunoglobulin content.

An alternative method of intubation which has recently been satisfactorily employed will utilize the Olympus flexible fiberoptic bronchoscope. After local anesthetic, this instrument is passed through the nose and into the bronchial tree using direct vision. Lavage is conducted after the tip is near a segmental bronchus, with 200 to 250 ml introduced and collected in aliquots. Because of the reliability and relative ease of intubation, this procedure will be increasingly used.

After the collection of cells by centrifugation, the number of cells obtained will be determined by standard hemocytometer techniques. An aliquot of cells will be stained supravitaly with neutral red-Janus green dyes, and the morphology of the cells will be noted. Particular attention will be paid to the cell size and the number and appearance of the granules. Cell viability will be tested by the exclusion of trypan blue dye.



The methods and the experimental approaches applicable to each of the specific aims outlined in the preceding section will be systematically discussed.

(1) Phagocytosis by the human macrophage. Using bacteria grown in broth cultures, in vitro phagocytosis and killing of organisms by macrophages from smokers and nonsmokers will be evaluated.

The evaluation of the ability of macrophages to ingest and kill bacteria will employ techniques similar to those used for peripheral blood leukocytes. Several different strains of potentially pathogenic bacteria will be used. Incubations will be conducted in tissue culture flasks rather than in tubes, and the sampling will be conducted over a period of time (usually three hours). Observations will be made using different combinations of fresh, heat-inactivated, and absorbed sera to investigate the requirements of antibodies and other serum factors in the opsonization of bacteria for phagocytosis by macrophages.

(2) Responsiveness to Chemotaxis. A modification of the Boyden chamber (J. Exp. Med. 115: 453, 1962) will be used to test the pulmonary macrophages for responsiveness to chemotactic stimuli. The bottom compartment consists of a 2 dram plastic medication vial, in which the solutions containing bacterial filtrates, casein, or other substances with known chemotactic activity are placed. The top compartment, which contains the suspension of macrophages, is a 100 x 13 plastic disposable test tube (Falcon No. 2027) with the bottom sawed off smoothly. A millipore filter of 8  $\mu$  mean pore diameter and thickness of 160  $\mu$  is cemented to the end of the tube.

Macrophages are suspended in buffer so that  $5 \times 10^5$  cells are introduced into the upper reaction chamber. The desired reactants (bacteria or bacterial filtrate preparations) are placed in the lower chamber and the whole assembly is incubated for 2 or more hours at 37°C. The filters are carefully cut away from the chamber, fixed and stained. The number of macrophages migrating completely through the filter is quantitated by counting the total in ten 400 X microscopic field. Appropriate controls will be run with each test so that results may be adjusted for spontaneous migration.

(3) Effects of lymphocyte migration-inhibition factor (MIF). Volunteers who are nonsmokers or cigarette smokers will be skin tested for the presence of delayed hypersensitivity to PPD, histoplasmin, Candida antigen, and other suitable reactants. Macrophages lavaged from these volunteers will be mixed with autologous lymphocytes obtained from the peripheral blood by separation of mixed leukocytes on glass-wool columns, according to the methods of Rocklin et al (11). Cells will be compacted into one-millimeter glass capillary tubes and the pelleted cells will be incubated in a coverslip chamber, with or without specific antigen. The inhibition of migration



is computed by calculating the areas of the fan-like advancing cell population. The area of the cells in the system with lymphocytes, macrophages, and antigen is compared to control systems in which one of the components is omitted.

Lymphocytes collected from a single donor and suspended in buffer will be incubated for up to 72 hours and the supernatants collected and tested for MIF production. This preparation will allow repeated testing using the same MIF and antigen over a series of observations. Guinea pig oil-induced macrophages will be used as well, to compare the responsiveness of heterologous and autologous macrophages to MIF from human lymphocytes and to serve as a positive control system for the MIF assay.

Morphological studies will include several different techniques, each of which will provide complementary information. These studies will include:

(1) Morphologic and biochemical studies of macrophage organelles. Macrophages lavaged from smokers and nonsmokers will be studied unstained by phase-contrast and phase-interference microscopy. Preparations will be viewed under fluorescence microscopy for quantitation of the amount of autofluorescent cytoplasmic inclusion material present. Representative samples will be fixed in glutaraldehyde and processed for electron microscopy, so that the ultrastructure of the macrophages obtained in these studies may be compared to those described by other investigators.

The macrophages will be lysed in 0.2 M sucrose and the lysate will be processed by differential centrifugation and sucrose gradient separation. The fractions obtained will be examined microscopically for the presence of the fluorescent granules evident in the intact cells. This material will be isolated and washed, then characterized using thin layer chromatography and other biochemical methods for examining lipid and protein content.

The sucrose-gradient fractions from the lysed macrophages will be examined for the content of acid phosphatase (measured by the liberation of phosphate from  $\beta$ -glycerophosphate) and cathepsin-D (quantitated by the release of Lowery chromogens from denatured hemoglobin). Latency of the enzymes will be studied by re-centrifuging the fractions and measuring the percentage of the total enzymes in the supernatant phase.

In these studies; the macrophages from smokers and nonsmokers will be compared, conserving the quantity and nature of the subcellular acid hydrolase distribution.



(2) Microspectrophotometric analyses: Macrophages obtained from normals, from cigarette smokers, and from subjects with experimental viral infections will be placed on slides under quartz cover slips and scanned using the Zeiss microspectrophotometer according to the methods of Weid et al (12, 13). Information about each 0.5 micron square segment of the cells will be stored in the PDP-12 Computer. After a sizeable population has been scanned (50 to 150 cells or more), characteristics may be compared by retrieving stored data from the computer and displaying the group or individual observations. Originally, the above techniques were designed for lymphocytes (12, 13) and have been extensively applied by MSC as a part of their early detection of disease program. However, recently the same techniques have successfully been used to study changes in macrophages (14).

The nature of the fluorescent material in the cytoplasm of macrophages from smokers will be investigated, to evaluate absorption maxima and relative content in relation to the smoking history. Also, measurements of DNA content of macrophages serially obtained from the same subject before, during and after viral infection will be of great interest.

### III. Background Work Performed:

The immunologic aspects of virus infections are not well understood. The effect of rubeola virus infection on the tuberculin skin test was perhaps the first observation of the ability of viruses to alter immune mechanisms (15, 16). This phenomenon remained primarily a curiosity until the congenital rubella epidemic in 1964-1965, when Alford (11) reported that these congenitally infected subjects continue to excrete virus for prolonged periods of time, a discovery which rekindled interest in virus-host interactions. Demonstration that this prolonged virus excretion occurred in spite of antibody production by the infected host ruled out "tolerance" as the mechanism of persistence and directed attention to altered cellular immune mechanisms as a possible explanation. Olson, South, and Good (18) in 1967 reported that rubella-infected patients showed decreased responsiveness of their peripheral lymphocytes to phytohemagglutinin stimulation. This unresponsiveness was temporary; when the patient's general condition improved, the PHA response became normal. Several other investigators have since reported similar findings (19, 20, 21). This phenomenon of unresponsiveness was investigated by Montgomery et al (22) who demonstrated that normal peripheral lymphocytes could be rendered unresponsive to PHA by the addition of live rubella virus in vitro. Several other viruses have since been shown to inhibit PHA response of peripheral lymphocytes in vivo and in vitro (23, 24).



In our laboratory, culture techniques are currently in use to determine the normal range of response of lymphocytes to PHA, poke weed mitogen, and streptolysin-O for normal subjects. The blastogenic response is being correlated with results of cells stained for DNA-RNA content and assayed by cytofluorometry. Preliminary findings indicate such correlations are valid and yield complementary data.

To determine the distribution of specific immunoglobulins on the surface of lymphocytes, trial experiments have begun with the aim of evaluating the specificity of commercially available antisera tagged with fluorescein.

As indicated previously, a number of investigators have found changes occurring in cells other than lymphocytes as a result of an infecting virus (5, 6, 7, 8, and 9). These individual viruses may be detected very early by use of immunofluorescence. The techniques are available for easy determination of the appearance of early antigens. In our laboratory, extensive work has been done utilizing immunofluorescent techniques in which antisera against infectious agents other than viruses have been required. These antisera were prepared by us and purified to react specifically with the infectious bacterium against which it was made. Such absorptive and purification techniques as will be needed for preparation of specific antiviral antisera are operative.

A third cell type which may be affected directly or may respond during infections is the macrophage. Studies have started to appear in which human alveolar macrophages obtained by saline lavage are being examined morphologically and functionally. Representative of these is the work reported by Harris et al (25) in which cells from smokers and nonsmokers were compared in their phagocytic ability, glucose utilization, and ultrastructural morphology. There was no impairment of phagocytosis and bacterial killing in cells from smokers compared to those of nonsmokers, in contrast to the findings of Green and Carolin (26) using *in vitro* exposure of rabbit alveolar macrophages to cigarette smoke in which phagocytosis is impaired. A higher rate of glucose utilization was detected in macrophages from cigarette smokers. The morphologic abnormalities previously noted by others were confirmed, especially the presence of increased numbers of residual bodies in cells from smokers.

In another study of human macrophages, Cohen and Cline examined the phagocytic abilities of macrophages maintained in culture after collection (27). These authors did not detect any differences in phagocytic ability of macrophages from 10 normal subjects, 5 tobacco smokers, and 6 marijuana smokers, in tests conducted within one day of collection of the cells.



In our laboratory, broncho-pulmonary saline lavage has been performed on 32 healthy male volunteers, ranging from 18 to 46 years of age. The volunteers were intubated with a 19-French balloon catheter and 250 ml of saline was introduced and removed in aliquots. Of the cells obtained, over 90% were alveolar macrophages. The 24 subjects on whom complete data has already been obtained included 13 non-smokers and 11 cigarette smokers with individual cigarette consumption ranging from 0.7 to 44 pack years.

The macrophages obtained by lavage were examined using light and fluorescence microscopy. These morphological studies confirmed previous observations that the macrophages from smokers contain large amounts of dense cytoplasmic inclusion material. Cells with abundant cytoplasmic inclusions, at times sufficiently numerous to obscure the nucleus of the cell, were seen in large numbers in the lavage from smokers. However, cells with numerous granules were also encountered in smaller numbers in lavage material from nonsmokers. The difference seemed to be quantitative rather than qualitative. The macrocytoplasmic granules were found to have intense yellow autofluorescence. Again, these autofluorescent granules were found in cells from nonsmokers, but were much more abundant in cells lavaged from cigarette smokers.

The fluorescent cytoplasmic material has not yet been isolated and further studied. It may be that the material is analogous to lipofuchsin pigments, which are insoluble, cross-linked lipids that cannot be completely degraded by enzymes in phagocytic vacuoles of cells. Such material may be stored within residual bodies for a considerable time. Alternatively, the stored material could represent such potential carcinogens as benzo-pyrene or other fluorescent byproducts of cigarette smoke.

In addition to the morphological studies, lysosomal enzymes were measured on the lavage material obtained from the 24 volunteers (28). The cells obtained by lavage were separated by centrifugation and the cell pellet was resuspended in saline. After cell counts were performed, the cells and the lysosomes were disrupted by sonication. The enzyme content for 4 representative lysosomal enzymes was measured. The results of the enzyme measurements, expressed as enzyme units per  $10^6$  cells, are presented in Table I. These data are given separately for the 13 control subjects (nonsmokers), for the 4 smokers of less than 1 pack of cigarettes daily, and for the remaining 7 smokers of 20 or more cigarettes per day. The mean acid phosphatase levels of the heavy smokers were elevated more than 5 times over the control levels, the differences being significant with a P value of  $< .02$  ("t" test). Similarly, the mean cathepsin levels of cells from heavy smokers were 6-fold increased over controls with a P value of  $< .01$ .  $\beta$ -glucuronidase was also elevated in the total group of 11



smokers, with a P value of  $< .05$ . There was a tendency for muramidase to be elevated with increased cigarette smoking, but the differences were borderline significance with P values of between .05 and .10.

Table I  
Cell-associated Enzymes in Macrophages from  
Nonsmokers and Cigarette Smokers:  
Enzyme Activities per  $10^6$  Cells

| Enzyme                  | Nonsmokers        | Cigarette Smokers          |                                        |
|-------------------------|-------------------|----------------------------|----------------------------------------|
|                         |                   | A<br>( $< 20/\text{day}$ ) | B<br>( $20 \text{ or } >/\text{day}$ ) |
| Acid phosphatase*       | $0.61 \pm 0.27$   | $1.83 \pm 1.37$            | $3.13 \pm 1.09$                        |
| Cathepsin*              | $0.83 \pm 0.26$   | $1.91 \pm 1.24$            | $5.85 \pm 2.84$                        |
| $\beta$ -Glucuronidase* | $32.96 \pm 10.79$ | $151.88 \pm 71.28$         | $101.05 \pm 48.65$                     |
| Muramidase**            | $16.98 \pm 8.06$  | $32.88 \pm 24.88$          | $38.75 \pm 15.13$                      |

\*Reaction products measured after incubation with substrate for 1 hour at  $38^\circ\text{C}$ .

\*\*Measured as time required for a 25% reduction in absorbance of a *Micrococcus lysodeikticus* suspension at  $25^\circ\text{C}$ . Expressed as egg white lysozyme equivalents.

The data obtained thus far suggests several interesting but very speculative conclusions. First of all, the presence of large amounts of fluorescent material in cytoplasmic granules of alveolar macrophages from cigarette smokers is not surprising, since previous investigators have noted discrete collections within phagosome-like organelles. In electron microscopic studies, the cytoplasmic inclusions were said by Pratt et al (29) to be found characteristically in macrophages from smokers and absent in cells from nonsmokers. The present studies demonstrated that macrophages from nonsmokers also contain distinct cytoplasmic granules which fluoresce, but a smaller percentage of cells show this abnormality. If these morphological changes are due to phagocytosis of inhaled substances by the macrophages, then the presence of these abnormalities in nonsmokers may reflect exposure of the volunteers in our population to urban air pollutants that are not abundant in New Mexico, the area from which Pratt's subjects came.

The other speculative conclusion which may be postulated from the data obtained thus far relates to the findings that macrophages and total lavage materials (cells and supernatant) from smokers contain higher concentrations of lysosomal enzymes than similar materials from nonsmokers. The 3 enzymes that were clearly elevated in smokers



(acid phosphatase,  $\beta$ -glucuronidase and cathepsin) are true acid hydrolases and are found in lysosomes of cells. Mucamidase, which was not as clearly altered in smokers, is present in abundant amounts in external secretions. It is not a true acid hydrolase, and it may be present in cytoplasm as well as lysosomes of cells. In addition to the acid hydrolases measured, one may speculate that other enzymes may be increased that could have as their substrate connective tissue, elastic tissue or other bronchial or alveolar constituents. An increase in enzymatic levels could be important to the pathogenesis of degenerative pulmonary diseases related to cigarette smoking.

The Boyden chamber technique of evaluating the responsiveness of cells to chemotactic stimuli has been employed successfully in studies of the differential responsiveness of polymorphonuclear leukocytes and mononuclear cells by Keller and Sorkin (30). A number of recent investigations have appeared, employing human polymorphonuclear cells, but studies of the responsiveness of the human macrophage to chemotaxins has been handicapped previously by a lack of readily-available cells for the in vitro assay.

We have made preliminary studies of responsiveness of human alveolar macrophages to chemotaxis, using casein hydrolysate and/or a broth culture of Serratia marcescens as the chemotaxis-promoting agents. The smaller, less-granulated macrophages have been found to be responsive, but only after the chambers have been incubated for a very prolonged time (12 to 24 hours).

A reproducible assay for delayed hypersensitivity in man, using guinea pig macrophages and human blood lymphocytes, has been described by Rocklin et al (11). These studies have demonstrated that the soluble migration inhibitory factor (MIF) from supernatants of lymphocytes incubated with antigen is immunologically specific, and that the assay may be made to work even though the indicator macrophages are from a different species than the lymphocyte donors.

We have been able to duplicate the observations using guinea pig macrophages and candida antigen MIF. In a small number of observations on human alveolar macrophages, there is a delay in migration from the capillary tube of 2 to 3 days, after which only a subpopulation of the smaller mononuclear cells migrate. These cells remain viable (excluding trypan blue and spreading activity on the glass) for up to 10 days, but migration does not continue after the first 5 days.



Pfitzer (14) has recently applied cytophotometric techniques to alveolar macrophages of normal individuals and one carcinogenic subject. DNA analyses using microspectrophotometer suggested that changes occurred in the composition of these cells which were distinguishable by use of this instrument. Cytoplasmic changes may also have occurred but were not examined.



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2. Dates of Employment: Sept. 1964 to Sept 1969

Position: Graduate Student

Employer: Baylor College of Medicine, Houston, Texas.

Description of Work:

During these five years, I studied as a graduate student in the Department of Microbiology of Baylor College of Medicine. I learned the basic techniques of diagnostic microbiology. My work for the Master of Science degree consisted of a study of the biochemical and structural properties of the cell wall of Haemophilus vaginalis 594. This project allowed me to develop techniques for growing fastidious microorganisms in large quantities, to harvest and fractionate the organisms, and to study the biochemistry of the isolated cell walls. The biochemical analyses included determinations of teichoic acid, mucopeptide, and amino acid contents of the cell wall. I also learned techniques of electron microscopy during extensive studies of the fine structure of the organism.

Work for my doctorate consisted of study of the immunity that develops in mice infected with Plasmodium berghei. The study was designed to show what effect humoral factors and antibody had upon parasitized erythrocytes both in the presence and absence of macrophages. The study provided me with an extensive background in the use and manipulation of animals, including techniques of implantation of millipore chambers into the peritoneal cavities of mice. I also became familiar with immunological procedures such as immunoelectrophoresis, gel diffusion, preparation and purification of serum proteins, density gradient ultracentrifugation, labeling gamma globulin, etc. In addition to learning the techniques needed to perform the above analyses, I have also learned to collect and critically evaluate scientific data and to use this data to plan subsequent experiments.

For two years I also assisted in teaching medical students in laboratories that were part of a course in clinical microbiology at Baylor College of Medicine.

3. Dates of Employment: July, 1963 to June, 1964

Position: Apprentice Technician

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Description of Work:

I completed one year of training in medical technology and became a registered medical technologist (ASCP-MT).



Society Memberships and Honors:

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Research Interests:

Clinical microbiology, immunology, bacterial genetics, and molecular biology with applications toward problems involving infectious diseases.

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Papers:

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5. Criswell, B.S., T. Rogers, and C.L. Fischer: The Lymphocyte, a possible means for detecting prodromal disease. (In preparation).
6. Criswell, B.S. and V. Knight: The possible role of the circulating and bound splenic lymphocytes in humoral immunity to murine Malaria. (In preparation).



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1. Criswell, B.S., C.D. Dukes, H.L. Gardner, C.L. Ladwig, and S.H. Black: Haemophilus vaginalis, a Cause of Vaginitis. Bacteriol. Proceed. for Amer. Soc. Microbiol., p. 97 (1967). Presented at the National Meeting of the American Society for Microbiology in New York, 1967.
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#### Thesis and Dissertation Titles:

1. Thesis: Criswell, B.S.: Structural and Chemical Analysis of Haemophilus vaginalis 594. Master's thesis. Baylor University College of Medicine, June, 1968.
2. Dissertation: Criswell, B.S.: Interaction of Humoral and Cellular Factors of Immunity in Murine Malaria. Doctoral dissertation. Baylor College of Medicine, June, 1969.



## CURRICULUM VITAE

Name: Vernon Knight

Date and place of birth: September 6, 1917 Osceola, Missouri

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Education:

1939 A.B. William Jewell College, Liberty, Missouri  
1943 M.D. Harvard Medical School, Boston, Massachusetts

Chronology of employment:

1943 Intern, Massachusetts Memorial Hospital, Boston, Mass.  
1946 - 1954 Assistant in Medicine to Assistant Professor,  
Cornell University Medical College, New York  
1954 - 1959 Associate Professor of Medicine, Vanderbilt University  
School of Medicine, Nashville, Tennessee  
1959 - 1966 Clinical Director, National Institute of Allergy and Infectious  
Diseases, National Institutes of Health, Bethesda, Md.  
1966 - Professor and Chairman, Department of Microbiology,  
Professor of Medicine, Baylor College of Medicine,

Board Certification: American Board of Internal Medicine, 1951

Military service: Jan. 10, 1944 to Sept. 25, 1946  
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Current appointments:

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|-------------------------------------|-------------------------------------------------------------|
| Baylor College of Medicine, Houston | Chairman, Curriculum Committee                              |
| Methodist Hospital, Houston         | Senior Attending Physician                                  |
| Ben Taub General Hospital, Houston  | Attending Physician                                         |
| University of Alabama, Birmingham   | Permanent Visiting Committee,<br>Department of Microbiology |



Current appointments -- continued

|                                                                                                                      |                                                                                               |
|----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| Grayson Foundation<br>Lexington, Kentucky                                                                            | Scientific Advisory Committee                                                                 |
| Journal of the American Medical Assoc.<br>Chicago, Illinois                                                          | Editorial Consultant                                                                          |
| U. S. Department of Defense<br>Armed Forces Epidemiological Board<br>Washington, D. C.                               | Commission on Epidemiological Survey<br>Commission on Influenza                               |
| U. S. Army Biological Laboratories<br>Frederick, Maryland                                                            | Consultant to Fort Detrick                                                                    |
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Professional societies:

AOA, Beta Chapter  
American Clinical and Climatological Association  
American College of Physicians  
American Federation for Clinical Research  
American Medical Association  
American Society for Clinical Investigation  
American Society for Microbiology (National and Texas Branch)  
~~American Thoracic Society~~  
Association of American Physicians  
Harris County Medical Society  
Infectious Diseases Society of America  
International Leprosy Association  
Society for Experimental Biology and Medicine  
Society of Sigma Xi  
Southern Society for Clinical Investigation  
Texas Medical Association

Other:

Who's Who in America  
American Men of Science



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20. Martin, R. R. and White, A.: Quantitative Nasal Culture: A Tool in Antibiotic Research. *Applied Microbiology* 22:397, Sept. 1971.



## ABSTRACTS

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G L E N N A. W A R R

123 Winkler Dr. #24 Houston, Texas 77017 A.C. 712-645-1248

Education Master of Science in Microbiology, Idaho State University, 1968.  
Bachelor of Science, Idaho State University, 1966. Major: Microbiology. Chemistry minor. Experience with immunology, virology, bacteriology, and analytical chemistry laboratory techniques. Research: Germination mechanisms in bacterial endospores.

Research, Instruction, practical experience with laboratory techniques and supervision Employed as Laboratory Assistant for seven months with the Microbiology Department of Idaho State University. Duties: Laboratory equipment operation and maintenance, equipment sterilization, culture media preparation, and culture isolation, propagation and preservation.

Following three years, 1965 to 1968, employed as Laboratory Instructor and Research Assistant with the Department of Microbiology at Idaho State University. Duties included supervision of two junior laboratory instructors, preparation, demonstration and discussion of laboratory exercises, as well as preparation and correction of student exercises. Research: Isolation, culturing and osmotic studies on Photobacteria; Gas Chromatography determination of fermentation products from bacteria in the family of Enterobacteriaceae. Preliminary research on genetic transformation in endospore germination utilizing Bacillus DNA.

practical experience in industry Employed as Head Bacteriologist and Research Chemist with Idaho Potato Starch Company. I established laboratory routines and procedures for examining dehydrated food products for Salmonella sp. and rope spores. Supervised technicians in quality control laboratory, and conducted research on product development and quality control.

Military Service, Nov. 1968 to Jan. 1971, - Line Officer in Navy - Weapons Officer on a Submarine Tender. Completed courses in clinical laboratory procedures, and Oceanography.

experience in biomedical research Employed as Research Assistant, Feb. 1971 to present, in the Infectious Diseases section of the Department of Medicine at Baylor College of Medicine. Duties include supervision of laboratory technicians in performance of laboratory techniques; devising and conducting applied and basic research experiments; and maintaining all laboratory supplies. Areas of research include: Biological and biochemical properties of human alveolar macrophage, immunological interactions between macrophage and lymphocytes, effects of antibiotics on human peripheral leukocyte chemotaxis and associated phenomena (publication in press), development of specific leukocyte chemotaxis and immunological activities following mycoplasma and influenza infections in humans (publications in press).

Personal data Born September 26, 1944...Married (no children)...height: five, eleven ...175 pounds...health excellent



## V. Facilities Statement

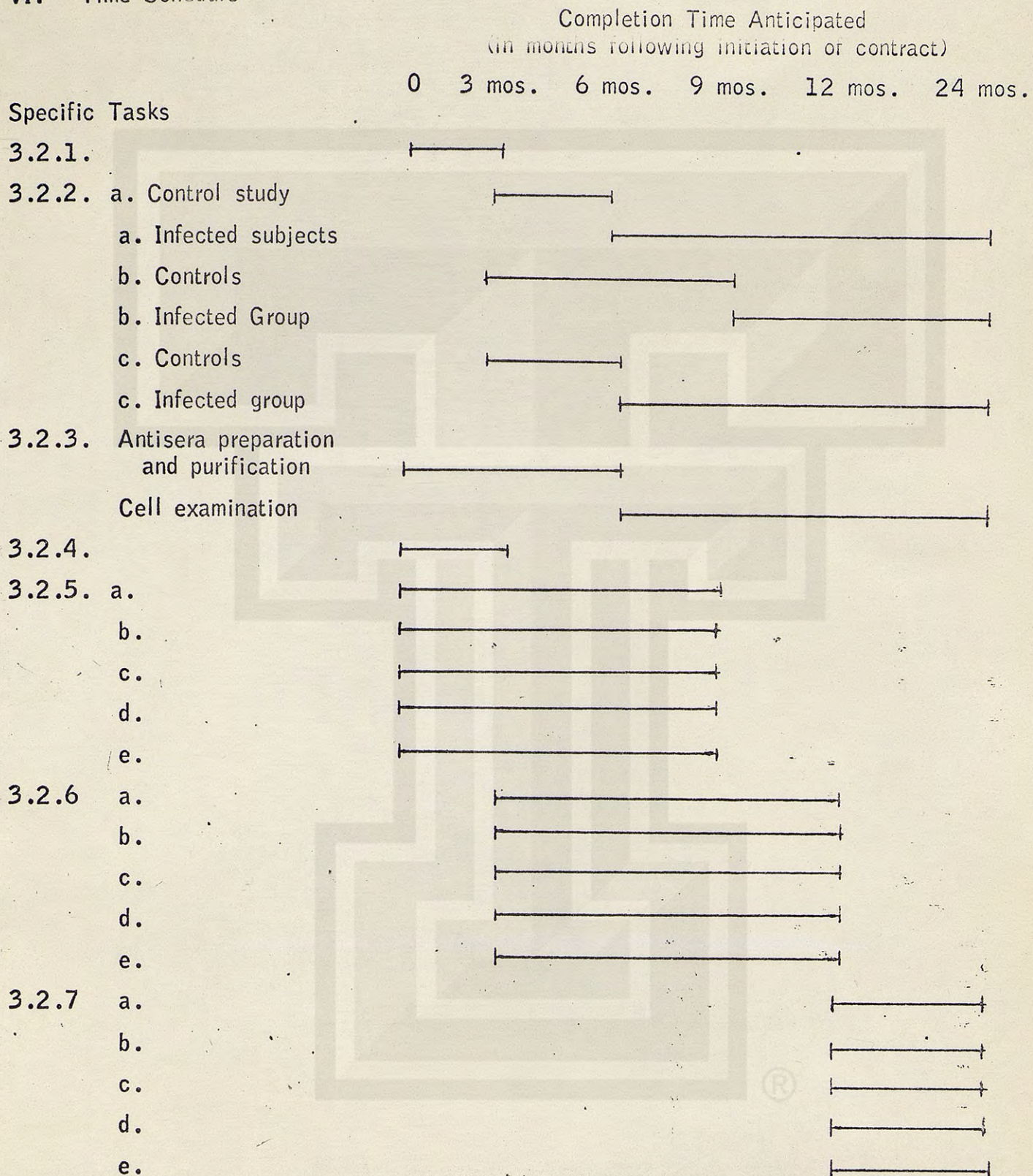
The laboratories for this research at Baylor College of Medicine are located in room 567-E of the Jones Building. Three laboratory modules with a total of 800 square feet will be used for these projects. In addition, there is an office-laboratory of 140 square feet in the Ben Taub General Hospital that is suitable for small projects.

Major equipment available includes a Beckman J-21 high speed refrigerated centrifuge; two incubators; microscopes adapted for brightfield, dark-field, phase, phase-fluorescence, phase-interference microscopy, and photomicrography; An American Instrument Company spectrophotofluorometer (model #125); a Shimadzu AUV-50 spectrophotometer; a Biosonik III ultrasonic probe disintegrator; and water baths, desktop centrifuges, an ultra-low temperature freezer, and other assorted small items. In addition, the Department of Medicine has established a common instrument room, autoclave and washroom facilities nearby on the same corridor in which additional major equipment suitable for shared use by several investigators is kept. This equipment includes a lyophilizer, a Beckman ultracentrifuge, a scintillation counter, and a liquid nitrogen storage chest. Quarters for small animals are available in the vivarium, one floor below the infectious diseases laboratory.

The Virus Diagnostic Laboratories at Methodist Hospital consist of four modules located on the fifth floor of the Fondren-Brown Building that are equipped for usual laboratory purposes. In addition, there are modern animal facilities on the same floor available for use of the laboratories.



## VI. Time Schedule





# VOLUME II

## BUDGET

### A. Category Requirements

| Budget for 3.2.1, 3.2.2a-c, 3.2.3, and 3.2.4                                                                                                                                                                   | Year 1 | Year 2 | Total |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|--------|-------|
| 1. Personnel: (5.5% increase for year 2)                                                                                                                                                                       |        |        |       |
| A. B. S. Criswell, Ph.D.                                                                                                                                                                                       | 18 737 | 19 768 |       |
| Fringe Benefits                                                                                                                                                                                                | 2 357  | 2 440  |       |
| Baylor Indirect Costs: (23.68% off Campus Salaries)                                                                                                                                                            | 4 437  | 4 681  |       |
| B. Research Associate                                                                                                                                                                                          | 11 000 | 11 605 |       |
| Fringe Benefits                                                                                                                                                                                                | 961    | 975    |       |
| Baylor Indirect Costs: (one-half salary at 40.98% on Campus Salaries; one-half salary at 23.68% off Campus Salaries.)                                                                                          | 2 254  | 2 378  |       |
|                                                                                                                                                                                                                | 1 302  | 1 374  |       |
| C. Research Assistant                                                                                                                                                                                          | 10 000 | 10 550 |       |
| Fringe Benefits                                                                                                                                                                                                | 938    | 950    |       |
| Baylor Indirect Costs: (one-half salary at 40.98% on Campus Salaries; one-half salary at 23.68% off Campus Salaries.)                                                                                          | 2 049  | 2 162  |       |
|                                                                                                                                                                                                                | 1184   | 1249   |       |
|                                                                                                                                                                                                                |        |        |       |
| TOTAL SALARIES + BENEFITS                                                                                                                                                                                      | 43 993 | 46 288 |       |
| TOTAL BAYLOR INDIRECT COSTS                                                                                                                                                                                    | 11 226 | 11 844 |       |
| 2. Minor Equipment:                                                                                                                                                                                            |        |        |       |
| A. 2 Revco Freezers to store specimens (1 per yr)                                                                                                                                                              | 4 000  | 4 000  |       |
| B. 1 National Tissue Culture Incubator                                                                                                                                                                         | 1 980  | -0-    |       |
| 3. Expendables and Laboratory Supplies: Media and reagents for tissue cultures, disposable glassware, fluorescent labelled antisera, film for photography, isotopes, animals for preparation of antisera, etc. | 6 000  | 5 000  |       |



## BUDGET CONTI.

|                                                                                                                           | Year 1 | Year 2 | Total   |
|---------------------------------------------------------------------------------------------------------------------------|--------|--------|---------|
| 4. Publication Expenses for papers and reports: Secretarial assistance, supplies, duplication, photography expenses, etc. | 1 500  | 1 500  |         |
| 5. Travel                                                                                                                 | 1 500  | 1 500  |         |
| TOTAL COST FOR SECTIONS 3.2.1, 3.2.2a-c, 3.2.3, and 3.2.4.                                                                | 70 199 | 70 132 | 140 331 |

| Budget for 3.2.5a-e, 3.2.6, and 3.2.7.                                                                                                | Year 1 | Year 2 | Total |
|---------------------------------------------------------------------------------------------------------------------------------------|--------|--------|-------|
| 1. Personnel: (5.5% increase for Year 2).                                                                                             |        |        |       |
| Research Assistant (Glenn A. Warr)                                                                                                    | 11 000 | 11 605 |       |
| Fringe Benefits                                                                                                                       | 961    | 975    |       |
| Baylor Indirect Costs: (one-half salary at 40.98% on Campus Salaries; one-half salary at 23.68% off Campus Salaries.)                 | 2 254  | 2 378  |       |
|                                                                                                                                       | 1 302  | 1 374  |       |
| TOTAL SALARIES AND BENEFITS                                                                                                           | 11 961 | 12 580 |       |
| TOTAL BAYLOR INDIRECT COSTS                                                                                                           | 3 556  | 3 752  |       |
| 2. Minor Equipment:                                                                                                                   |        |        |       |
| A. MIF Chambers                                                                                                                       | 611    | 868    |       |
| B. Olympus bronchoscope                                                                                                               | 3 572  | -0-    |       |
| 3. Payment to Volunteers: (Increase in yr. 2 needed because an active infection is used and requires higher pay for each individual.) | 1 500  | 4 000  |       |
| 4. Expendables: Disposable glassware, reagents and media, film for photography and developing, millipore filters, etc.                | 3 000  | 3 000  |       |
| 5. Travel                                                                                                                             | 800    | 800    |       |



BUDGET CONTI.

|                                                     | Year 1 | Year 2 | Total   |
|-----------------------------------------------------|--------|--------|---------|
| TOTAL COST FOR SECTIONS 3.2.5a-e, 3.2.6, and 3.2.7. | 25 000 | 25 000 | 50 000  |
| TOTAL COSTS FOR CONTRACT                            | 95 199 | 95 133 | 190 331 |



## (RESEARCH AND DEVELOPMENT)

Budget Bureau No. 22-R100

This form is for use when (i) submission of cost or pricing data (see NASA PR 3,807-3) is required and (ii) substitution for the DD Form 633 is authorized by the contracting officer.

PAGE NO.

NO. OF PAGES

## NAME OF OFFEROR

Baylor College of Medicine

## HOME OFFICE ADDRESS (Include ZIP Code)

1200 HOURSUND  
HOUSTON, TEXAS 77025

## SUPPLIES AND/OR SERVICES TO BE FURNISHED

Both supplies and services are required to evaluate the cellular immune response and develop procedures for screening potential flight crews for early detection of diseases.

## DIVISION(S) AND LOCATION(S) WHERE WORK IS TO BE PERFORMED

Baylor College of Medicine, Houston, Tex.

## TOTAL AMOUNT OF PROPOSAL

\$ 190,331 for 2 yrs.

## GOVT SOLICITATION NO.

9-BB32-79-2-375P

## DETAIL DESCRIPTION OF COST ELEMENTS

| 1. DIRECT MATERIAL (Itemize on Exhibit A)                                          | EST COST (\$)   | TOTAL EST COST <sup>1</sup> | REFER- <sup>2</sup> ENCE |
|------------------------------------------------------------------------------------|-----------------|-----------------------------|--------------------------|
| a. PURCHASED PARTS                                                                 | 17 000          |                             | Ex. A                    |
| b. SUBCONTRACTED ITEMS                                                             |                 |                             |                          |
| c. OTHER - (1) RAW MATERIAL                                                        |                 |                             |                          |
| (2) YOUR STANDARD COMMERCIAL ITEMS                                                 |                 |                             |                          |
| (3) INTERDIVISIONAL TRANSFERS (At other than cost)                                 |                 |                             |                          |
| TOTAL DIRECT MATERIAL                                                              |                 | 17,000                      |                          |
| 2. MATERIAL OVERHEAD <sup>3</sup> (Rate % X \$ base = )                            |                 |                             |                          |
| 3. DIRECT LABOR (Specify)                                                          | ESTIMATED HOURS | RATE/HOUR                   | EST COST (\$)            |
| 1 Faculty (Salary + Benefits)                                                      | 40/wk           |                             | 43 302                   |
| 1 Research Associate (Salary + Benefits)                                           | 40/wk           |                             | 24 541                   |
| 2 Research Assistants (Salary + Benefits)                                          | 40/wk           |                             | 46 979                   |
| TOTAL DIRECT LABOR                                                                 |                 |                             | 114,822                  |
| 4. LABOR OVERHEAD (Specify department or cost center) <sup>3</sup>                 | O.H. RATE       | X BASE =                    | EST COST (\$)            |
| A. On Campus Salaries                                                              | 40.98%          | 32 880                      | 13 474                   |
| B. Off Campus Salaries                                                             | 23.68%          | 52 132                      | 16 904                   |
| TOTAL LABOR OVERHEAD                                                               |                 |                             | 30,378                   |
| 5. SPECIAL TESTING (Including field work at Government installations)              | EST COST (\$)   |                             |                          |
| Payment to Human Volunteers                                                        | 5 500           |                             |                          |
| TOTAL SPECIAL TESTING                                                              |                 | 5,500                       |                          |
| 6. SPECIAL EQUIPMENT (If direct charge) (Itemize on Exhibit A)                     |                 | 15 031                      | Ex. A, c, d, 1           |
| 7. TRAVEL (If direct charge) (Give details on attached Schedule)                   | EST COST (\$)   |                             |                          |
| a. TRANSPORTATION                                                                  |                 |                             |                          |
| b. PER DIEM OR SUBSISTENCE (For 4 investigators + 3 technicians)                   | 4 600           |                             |                          |
| TOTAL TRAVEL                                                                       |                 | 4,600                       |                          |
| 8. CONSULTANTS (Identify - purpose - rate) Co-investigators                        | EST COST (\$)   |                             |                          |
| Dr. V. Knight, Dr. R.R. Martin, and Dr. J.A. Kasel                                 | -0-             |                             |                          |
| TOTAL CONSULTANTS                                                                  |                 |                             |                          |
| 9. OTHER DIRECT COSTS (Itemize on Exhibit A)                                       |                 | 3 000                       | Ex. A, C, d, 1           |
| 10. TOTAL DIRECT COST AND OVERHEAD                                                 |                 |                             |                          |
| 11. GENERAL AND ADMINISTRATIVE EXPENSE (Rate % of cost element Nos. ) <sup>3</sup> |                 |                             |                          |
| 12. ROYALTIES <sup>4</sup>                                                         |                 |                             |                          |
| 13. TOTAL ESTIMATED COST                                                           |                 | 190,331                     |                          |
| 14. FEE OR PROFIT                                                                  |                 |                             |                          |
| 15. TOTAL ESTIMATED COST AND FEE OR PROFIT                                         |                 | 190,331                     |                          |

This proposal is submitted for use in connection with and in response to (Describe RFP, etc.)

and reflects our best estimates as of this date, in accordance with the instructions to offerors and the footnotes which follow.

## TYPED NAME AND TITLE

Joseph M. Merrill, M.D., Exec. Vice-President

## SIGNATURE

## NAME OF FIRM

Baylor College of Medicine

## DATE OF SUBMISSION

May 30, 1972



|                                                                                                                                                                                                                                 | EST. COST (\$) |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| 1.a. Sections 3.2.1, 3.2.2a-c, 3.2.3, and 3.2.4                                                                                                                                                                                 | 11 000         |
| Expendable laboratory supplies for 2 yrs: Media and reagents for tissue cultures, disposable glassware, fluorescent labelled antisera, film for photography and processing, isotopes, animals for preparation of antisera, etc. |                |
| Sections 3.2.5a-c, 3.2.6, and 3.2.7                                                                                                                                                                                             | 5 000          |
| Expendable laboratory supplies for 2 yrs: Disposable glassware, reagents and media for cell maintenance, film for photography and processing, millipore filters, etc.                                                           |                |
| 1. For breakdown on individual salaries see Vol. II--Budget--A.                                                                                                                                                                 |                |
| Category Requirements p.                                                                                                                                                                                                        |                |

HAVE THE DEPARTMENT OF DEFENSE, NATIONAL AERONAUTICS AND SPACE ADMINISTRATION, OR THE ATOMIC ENERGY COMMISSION PERFORMED ANY REVIEW OF YOUR ACCOUNTS OR RECORDS IN CONNECTION WITH ANY OTHER GOVERNMENT PRIME CONTRACT OR SUBCONTRACT WITHIN THE PAST TWELVE MONTHS?

☒ YES ☐ NO If yes, identify below.

NAME AND ADDRESS OF REVIEWING OFFICE (Include ZIP Code)

W. Audit Agency  
Box 143

Austin, Texas 78767

TELEPHONE NUMBER/EXTENSION

512- 377-5316

WILL YOU REQUIRE THE USE OF ANY GOVERNMENT PROPERTY IN THE PERFORMANCE OF THIS PROPOSED CONTRACT?

☒ YES ☐ NO If yes, identify on a separate page. (See Ex. A. Conti. Page 1.).

DO YOU REQUIRE GOVERNMENT CONTRACT FINANCING TO PERFORM THIS PROPOSED CONTRACT?

☒ YES ☒ NO If yes, identify: ☐ ADVANCE PAYMENTS ☐ PROGRESS PAYMENTS OR ☐ GUARANTEED LOANS

DO YOU NOW HOLD ANY CONTRACT (or, do you have any independently financed (IR & D) projects) FOR THE SAME OR SIMILAR WORK CALLED FOR BY THIS PROPOSED CONTRACT?

☒ YES ☐ NO If yes, identify

DOES THIS COST SUMMARY CONFORM WITH THE COST PRINCIPLES SET FORTH IN NASA PR, PART 15 (see 3.807-2(c)(2))?

☒ YES ☐ NO If no, explain on a separate page.

#### INSTRUCTIONS TO OFFERORS

The purpose of this form is to provide a standard format by which the offeror submits to the Government a summary of incurred and estimated cost (and attached supporting information) available for detailed review and analysis. Prior to the award of a contract resulting from this proposal the offeror shall, under the conditions stated in NASA PR 3.807-3, be required to submit a Certificate of Current Cost or Pricing Data (see NASA PR 3.807-3(c) and 3.807-4).

As part of the specific information required by this form, the offeror must submit with this form, and clearly identify as such, cost or pricing data (that is, data which is verifiable and fact-based and otherwise as defined in NASA PR 3.807-3(e)). In addition, the offeror must submit with this form any information reasonably required to explain the offeror's estimating process, including:

- the judgmental factors applied and the mathematical or other methods used in the estimate including those used in projecting from known data, and
- the contingencies used by offeror in his proposed price.

3. When attachment of supporting cost or pricing data to this form is impracticable, the data will be specifically identified and described (with schedules as appropriate), and made available to the contracting officer or his representative upon request.

4. The format for the "Cost Elements" is not intended as rigid requirements. These may be presented in different format with the prior approval of the contracting officer if required for more effective and efficient presentation. In all other respects this form will be completed and submitted without change.

5. By submission of this proposal, offeror, if selected for negotiation, grants to the contracting officer, or his authorized representative, the right to examine, for the purpose of verifying the cost or pricing data submitted, those books, records, documents and other supporting data which will permit adequate evaluation of such cost or pricing data, along with the computations and projections used therein. This right may be exercised in connection with any negotiations prior to contract award.

#### FOOTNOTES

Enter in this column those necessary and reasonable costs which in the judgment of the offeror will properly be incurred for efficient performance of the contract. When any of the costs in this column have already been incurred (e.g., on a prior contract or change order), describe them on an attached supporting schedule. Identify all sales and transfers between plants, divisions, or organizations under a common control which are included at other than the lower of cost to the original transferor or current market price.

When space in addition to that available in Exhibit A is required, attach separate pages as necessary and identify in the "Reference" column the attachment in which information supporting the specific cost element may be found. No standard format is prescribed; however, the cost or pricing data must be accurate, complete and current, and the judgment factors used in projecting from the data to the estimates must be stated in sufficient detail to enable the contracting officer to evaluate the proposal. For example, provide the basis used for pricing materials such as by vendor quotations, shop estimates, or price prices; the reason for use of overhead rates which differ significantly from experienced rates (reduced volume, a new major rearrangement, etc.); or justification for an increase in labor rates (anticipated wage and salary increases, etc.). Identify and explain any contingencies which are included in the proposed price, such as anticipated costs of rejects and rework, or anticipated technical difficulties.

3. Indicate the rates used and provide an appropriate explanation. Where agreement has been reached with Government representatives on the use of forward pricing rates, describe the nature of the agreement. Provide the method of computation and application of your overhead expense, including cost breakdown and showing trends and budgetary data as necessary to provide a basis for evaluation of the reasonableness of proposed rates.

4. If the total royalty cost entered here is in excess of \$250 provide on a separate page (or on DD Form 783, Royalty Report) the following information on each separate item of royalty or license fee: name and address of licensor; date of license agreement; patent numbers, patent application serial numbers, or other basis on which the royalty is payable; brief description, including any part or model numbers of each contract item or component on which the royalty is payable; percentage or dollar rate of royalty per unit; unit price of contract item; number of units; and total dollar amount of royalties. In addition, if specifically requested by the contracting officer, a copy of the current license agreement and identification of applicable claims of specific patents shall be provided.

5. Provide a list of principal items within each category indicating known or anticipated source, quantity, unit price, competition obtained, and basis of establishing source and reasonableness of cost.



EXHIBIT A Continued Page 1.

| COST EL NO. | Item Description                                                                                                                                                                          | Est Cost (\$) |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 6.          | Special Equipment<br>For Sections 3.2.1, 3.2.2a-c, 3.2.3, and 3.2.4.                                                                                                                      |               |
|             | A. 2 Revco Freezers for storage of viral specimens required specifically for this study. 1 freezer per year will be required.                                                             | 8 000         |
|             | B. 1 National Tissue Culture Incubator for work pertaining exclusively to this study will be required.                                                                                    | 1 980         |
|             | For Sections 3.2.5 a-e, 3.2.6, and 3.2.7.                                                                                                                                                 |               |
|             | A. MIF Chambers required for specific examination of macrophages for this study. Slightly more will be needed the second year than first because of increased work loads.                 | 1 479         |
|             | B. 1 Olympus bronchoscope for lavage of subjects in the macrophage study.                                                                                                                 | 3 572         |
| 9.          | Other Direct Costs:<br>Publication expenses for scientific papers and reports:<br>Secretarial assistance, supplies, duplication, graphics and photography expenses, etc. (1 500 per yr)   | 3 000         |
| 11.         | Specialized equipment within the Cellular Analysis Laboratory at the Manned Spacecraft Center will be required as stated in the Statement of Work 3.0 Technical Requirements 3.1 General. |               |



1. SMALL BUSINESS (See par. 14 on SF 33-A.)

He ☐ is, ☒ is not, a small business concern. If offeror is a small business concern and is not the manufacturer of the supplies offered, he also represents that all supplies to be furnished hereunder ☐ will, ☐ will not, be manufactured or produced by a small business concern in the United States, its possessions, or Puerto Rico.

2. REGULAR DEALER—MANUFACTURER (Applicable only to supply contracts exceeding \$10,000.)

He is a ☐ regular dealer in, ☐ manufacturer of, the supplies offered.

N/A

3. CONTINGENT FEE (See par. 15 on SF 33-A.)

(a) He ☐ has, ☒ has not, employed or retained any company or person (other than a full-time, bona fide employee working solely for the offeror) to solicit or secure this contract, and (b) he ☐ has, ☒ has not, paid or agreed to pay any company or person (other than a full-time bona fide employee working solely for the offeror) any fee, commission, percentage, or brokerage fee contingent upon or resulting from the award of this contract; and agrees to furnish information relating to (a) and (b) above, as requested by the Contracting Officer. (For interpretation of the representation, including the term "bona fide employee," see Code of Federal Regulations, Title 41, Subpart 1-1.5.)

4. TYPE OF BUSINESS ORGANIZATION

He operates as ☐ an individual, ☐ a partnership, ☐ a nonprofit organization, ☒ a corporation, incorporated under the laws of the State of

TEXAS

5. AFFILIATION AND IDENTIFYING DATA (Applicable only to advertised solicitations.)

Each offeror shall complete (a) and (b) if applicable, and (c) below:

(a) He ☐ is, ☒ is not, owned or controlled by a parent company. (See par. 16 on SF 33-A.)

(b) If the offeror is owned or controlled by a parent company, he shall enter in the blocks below the name and main office address of the parent company:

Name of Parent company and main office address \_\_\_\_\_

(include ZIP Code) \_\_\_\_\_

(c) Employer's identification number (See par. 17 on SF 33-A.) 74 1613878

(Offeror's E.I. No.)

(Parent Company's E.I. No.)

6. EQUAL OPPORTUNITY

He ☒ has, ☐ has not, participated in a previous contract or subcontract subject either to the Equal Opportunity clause herein or the clause originally contained in section 301 of Executive Order No. 10925, that he ☒ has, ☐ has not, filed all required compliance reports; and that representations indicating submission of required compliance reports, signed by proposed subcontractors, will be obtained prior to subcontract awards. (The above representation need not be submitted in connection with contracts or subcontracts which are exempt from the clause.)

7. BUY AMERICAN CERTIFICATE

The offeror hereby certifies that each end product, except the end products listed below, is a domestic source end product (as defined in the clause entitled "Buy American Act"); and that components of unknown origin have been considered to have been mined, produced, or manufactured outside the United States.

EXCLUDED END PRODUCTS

N/A

COUNTRY OF ORIGIN

8. CERTIFICATION OF INDEPENDENT PRICE DETERMINATION (See par. 18 on SF 33-A.)

(a) By submission of this offer, the offeror certifies, and in the case of a joint offer, each party thereto certifies as to its own organization, that in connection with this procurement:

(1) The prices in this offer have been arrived at independently, without consultation, communication, or agreement, for the purpose of restricting competition, as to any matter relating to such prices with any other offeror or with any competitor;

(2) Unless otherwise required by law, the prices which have been quoted in this offer have not been knowingly disclosed by the offeror and will not knowingly be disclosed by the offeror prior to opening in the case of an advertised procurement or prior to award in the case of a negotiated procurement, directly or indirectly to any other offeror or to any competitor; and

(3) No attempt has been made or will be made by the offeror to induce any other person or firm to submit or not to submit an offer for the purpose of restricting competition.

(b) Each person signing this offer certifies that:

(1) He is the person in the offeror's organization responsible within that organization for the decision as to the prices being offered herein and that he has not participated, and will not participate, in any action contrary to (a) (1) through (a) (3) above; or

(2) (i) He is not the person in the offeror's organization responsible within that organization for the decision as to the prices being offered herein but that he has been authorized in writing to act as agent for the persons responsible for such decision in certifying that such persons have not participated, and will not participate, in any action contrary to (a) (1) through (a) (3) above; and as their agent does hereby so certify; and (ii) he has not participated, and will not participate, in any action contrary to (a) (1) through (a) (3) above.

9. CERTIFICATION OF NONSEGREGATED FACILITIES

(Applicable to (1) contracts, (2) subcontracts, and (3) agreements with applicants who are themselves performing federally assisted construction contracts, exceeding \$10,000 which are not exempt from the provisions of the Equal Opportunity clause.)

By the submission of this bid, the bidder, offeror, applicant, or subcontractor certifies that he does not maintain or provide for his employees any segregated facilities at any of his establishments, and that he does not permit his employees to perform their services at any location, under his control, where segregated facilities are maintained. He certifies further that he will not maintain or provide for his employees any segregated facilities at any of his establishments, and that he will not permit his employees to perform their services at any location, under his control, where segregated facilities are maintained. The bidder, offeror, applicant, or subcontractor agrees that a breach of this certification is a violation of the Equal Opportunity clause in this contract. As used in this certification, the term "segregated facilities" means any waiting rooms, work areas, rest rooms and wash rooms, restaurants and other eating areas, time clocks, locker rooms and other storage or dressing areas, parking lots, drinking fountains, recreation or entertainment areas, transportation, and housing facilities provided for employees which are segregated by explicit directive or are in fact segregated on the basis of race, color, religion or national origin, because of habit, local custom, or otherwise. He further agrees that (except where he has obtained identical certifications from proposed subcontractors for specific time periods) he will obtain identical certifications from proposed subcontractors prior to the award of subcontracts exceeding \$10,000 which are not exempt from the provisions of the Equal Opportunity clause; that he will retain such certifications in his files; and that he will forward the following notice to such proposed subcontractors (except where the proposed subcontractors have submitted identical certifications for specific time periods):

Notice to prospective subcontractors of requirement for certifications of nonsegregated facilities.

A Certification of Nonsegregated Facilities must be submitted prior to the award of a subcontract exceeding \$10,000 which is not exempt from the provisions of the Equal Opportunity clause. The certification may be submitted either for each subcontract or for all subcontracts during a period (i.e., quarterly, semiannually, or annually). NOTE: The penalty for making false statements in offers is prescribed in 18 U.S.C. 1001.

| ACKNOWLEDGMENT OF AMENDMENTS                                                                                                       | AMENDMENT NO. | DATE | AMENDMENT NO. | DATE |
|------------------------------------------------------------------------------------------------------------------------------------|---------------|------|---------------|------|
| The offeror acknowledges receipt of amendments to the Solicitation for Offers and related documents numbered and dated as follows: |               |      |               |      |

NOTE—Offers must set forth full, accurate, and complete information as required by this Solicitation (including attachments). The penalty for making false statements in offers is prescribed in 18 U.S.C. 1001.



### Certification of Equal Employment Compliance

By submission of this offer, the offeror certifies that, except as noted below, up to the date of this offer, no advice, information or notice has been received by the offeror from any Federal Government Agency or representative thereof that the offeror or any of its divisions or affiliates or known first-tier subcontractors is in violation of any of the provisions of Executive Order No. 11246 of September 24, 1965, Executive Order No. 11375 of October 13, 1967, or rules and regulations of the Secretary of Labor (41CFR, chapter 60) and specifically as to not having an acceptable affirmative action program or being in noncompliance with any other aspect of the Equal Employment Opportunity Program. It is further certified and agreed that should there be any change in the status of circumstances certified to above between this date and the date of expiration of this offer or any extension thereof, the Government Contracting Officer cognizant of this procurement will be notified forthwith.

### Affirmative Action Program

(The following certification shall be completed by each offeror whose offer is \$50,000 or more and who has 50 employees or more)

The offeror certifies that he ☒ has, ☐ has not, developed and maintained at each of his establishments Equal Opportunity Affirmative Action Programs, pursuant to 41CFR60.2.



CERTIFICATE OF CURRENT COST OR PRICING DATA (OCTOBER 1969)

This is to certify that, to the best of my knowledge and belief, cost or pricing data as defined in NASA PR 3.807-3(e) submitted, either actually or by specific identification in writing (see NASA PR 3.807-3(f)), to the Contracting Officer or his representative in support of \_\_\_\_\_

RFP 9-BB32-79-2-375P

are accurate, complete, and current as of 30 May 1972 <sup>\*</sup>  
day month year <sup>\*\*</sup>

Firm Baylor College of Medicine

Name \_\_\_\_\_

Title Executive Vice President

5-30-72 <sup>\*\*\*</sup>  
Date of Execution

- \* Describe the proposal, quotation, request for price adjustment or other submission involved, giving appropriate identifying number (e.g., RFP No. \_\_\_\_\_).
- \*\* This date shall be the date when the price negotiations were concluded and the contract price was agreed to. The responsibility of the contractor is not limited by the personal knowledge of the contractor's negotiator if the contractor had information reasonably available (see 3.807-5(a)) at the time of agreement, showing that the negotiated price is not based on accurate, complete and current data.
- \*\*\* This date should be as close as practicable to the date when the price negotiations were concluded and the contract price was agreed to.



## RESUME

Name: Bennie Sue Cook Criswell

Date and place of birth: November 17, 1942; Huntsville, Texas.

Marital Status: Married, 1 child

Education:

| <u>Dates</u> | <u>Degree</u>       | <u>Institution</u>                             |
|--------------|---------------------|------------------------------------------------|
| 1960-64      | B.S.                | North Texas State University                   |
| 1963-64      | Med. Tech. Training | M. D. Anderson Hospital and<br>Tumor Institute |
| 1964-68      | M. S.               | Baylor Univ. Coll. of Medicine                 |
| 1968-69      | Ph.D.               | Baylor College of Medicine                     |

Work Experience:

1. October 1969 to July 1, 1972: Postdoctoral Fellow, Baylor College of Medicine, Houston, Texas, and MSC-NASA, Houston, Texas

July 1, 1972 - : Instructor, Baylor College of Medicine  
Houston, Texas.

Description of work:

For the past two years, I have worked as a postdoctoral fellow at the Manned Spacecraft Center. I have been responsible for the design and development of a program directed toward the early detection of diseases during the prodromal stages, that is detection prior to the development of clinical signs and symptoms. I have designed a protocol using animal models for the study of the cellular immune response during timed intervals following inoculation of a specific disease agent. To date two illnesses have been studied. Lymphocytes appear to provide the earliest cellular indication of an ensuing disease. Currently, the earlier findings are being applied to human work as a possible screening method for early disease states.