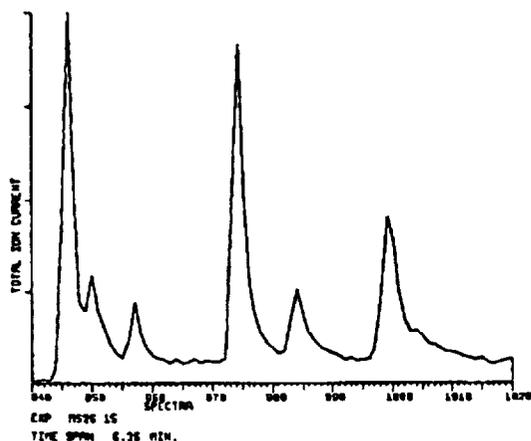


GLC CHART RECORD OF TFA - DIPEPTIDE METHYL ESTERS



A COMPUTER PLOT OF THE SUMMED PEAKS (IONS) OF EACH SPECTRUM

Figure 5. The above trace is a GLC flame ionization chart of an experiment. Below is a total ion plot, created, and plotted by the computer, that indexes the individual spectra

```

OPTION = ?TYPE
EXPERIMENT# = ?1526 15
FROM SPECTRUM = ?955
TO = ?960
BY MASS, AMP, or ENTIRE = ?AMP
FROM MASS = ?330
TO = ?750
NUM'BER = ?9
SPECTRUM 955
MASS 31 42 43 46 57 69 139 166 167
AMPL 103 22 22 26 22 30 23 37 27
SPECTRUM 956
MASS 31 45 46 69 70 96 139 166 167
AMPL 117 25 25 32 33 27 33 64 44
SPECTRUM 957
MASS 31 43 45 69 70 72 139 166 167
AMPL 124 38 39 57 64 52 47 102 76
SPECTRUM 958
MASS 31 43 45 69 70 72 139 166 167
AMPL 114 31 32 36 31 34 32 60 48
SPECTRUM 959
MASS 31 43 56 69 70 96 139 166 167
AMPL 116 26 24 26 27 25 29 51 39
SPECTRUM 960
MASS 31 32 45 46 69 70 72 166 167
AMPL 113 22 24 23 25 24 21 36 30
  
```

Figure 6. The principal peak listing from the matrix search

times the circuit transfer function over the frequency range in ω . The first shown below (on the left side of the equation) represents the white noise retained by a full integrator during a time interval T . The second expresses the white noise passed by a simple amplifier of bandpass, $f_{eq} = 1/(2\pi\tau_{eq})$.

$$\int_0^{\infty} \left| \int_0^T e^{j\omega t} dt \right|^2 d\omega = \int_0^{\infty} \left| e^{j\omega t} \frac{1}{1 + j\omega\tau_{eq}} \right|^2 d\omega \quad (3)$$

A solution for τ_{eq} may be made by numerical methods:

$$\tau_{eq} \cong 0.496 T \quad (4)$$

Using this value, it is found in the example that $f_{eq} = 40$ Hz.

A last analytical benefit of the system is the efficiency of detecting ions. In any MS with electronic ion detection, an ion is detected or not detected and there is no "5% detection."

There is a 5% probability an ion generated in the source will pass the analyzer and hit the detector. Or more accurately, if when a peak is "centered," n ions register upon the detector each millisecond, it may be expected that at the appropriate low side of the peak, $n/20$ ions will register each millisecond. The control method is always "centered" upon the peak, hence the total expected ions will be $T \times n$ ions. However, in scanning a gaussian peak, it can be shown that for an equivalent time, from the first 5% point through the maximum and out to the last 5% point, the total expected ions will be approximately $0.51 \times T \times n$, where now T is the time from 5% point to 5% point. These numbers of ions expected to register upon the detector are tabulated as "Ions Detected" in Table II. Many small, but important, peaks will have an n of 1 to 10 ions per millisecond under the operating conditions imposed by some modern research requirements.

A figure of merit for comparing the systems may be defined:

$$\text{Figure of Merit} = \frac{1000}{n} \times \frac{\text{Detected Ions}}{\text{Bandwidth (Hz)}} \quad (5)$$

This is the final tabulation in Table II. Our use of the system has verified these results, that introducing computer control to the quadrupole or T-o-F MS can enhance the useful sensitivity by a factor of 3 to 8. The greater convenience offered to the user is not at the expense of performance, but offers these intrinsic advantages.

One other practical benefit is possible by reviewing the attributes of resolution. The idealized gaussian peak considered here, which has a width commensurate with the 10% valley resolution criteria, has a contribution of only 0.06% at the neighboring integer mass position. Since in the control method, only the cross contribution at the integer positions is harmful for some experiments, the resolution may be degraded until a 0.5% or 1% cross contribution is observed. If the physics of the particular instrument are exploited, it will be found that this will greatly increase the portion of generated ions that will be passed by the analyzer.

Some consideration should be given here to the software and its interaction with the user and the instrument. A mode of conversation has been programmed into the system to prompt the user for necessary parameters, and then expand upon these parameters to conduct the necessary detailed operation of the instrument. In retrospect we find that we have very closely followed the concept of R. J. Spinard (21). A similar problem occurs with the presentation of information, which is often too voluminous for complete display. To overcome this, the bulk of the information is held in the computer, and is made available by similar conversational techniques.

It should be realized that the present software is in an embryonic state of development compared to its eventual potential. The system of computer, interface, and MS may be defined in terms of computer syntax to a systems developer in a manner comparable to the definition of a specific data processing task to a system programmer. There is an enormous potential in the implemented syntax of this instrumentation system for the chemist who may have special requirements and who understands fully the principles of the augmented instrument. The user can then program, or have programmed, efficient solutions to many of his instrumentation problems.

The features that do allow the foregoing benefits, do themselves impose certain limitations. The control system does not normally return any information about doubly ionized odd

(21) R. J. Spinard, *Science*, **158**, 3797, 55 (1967).

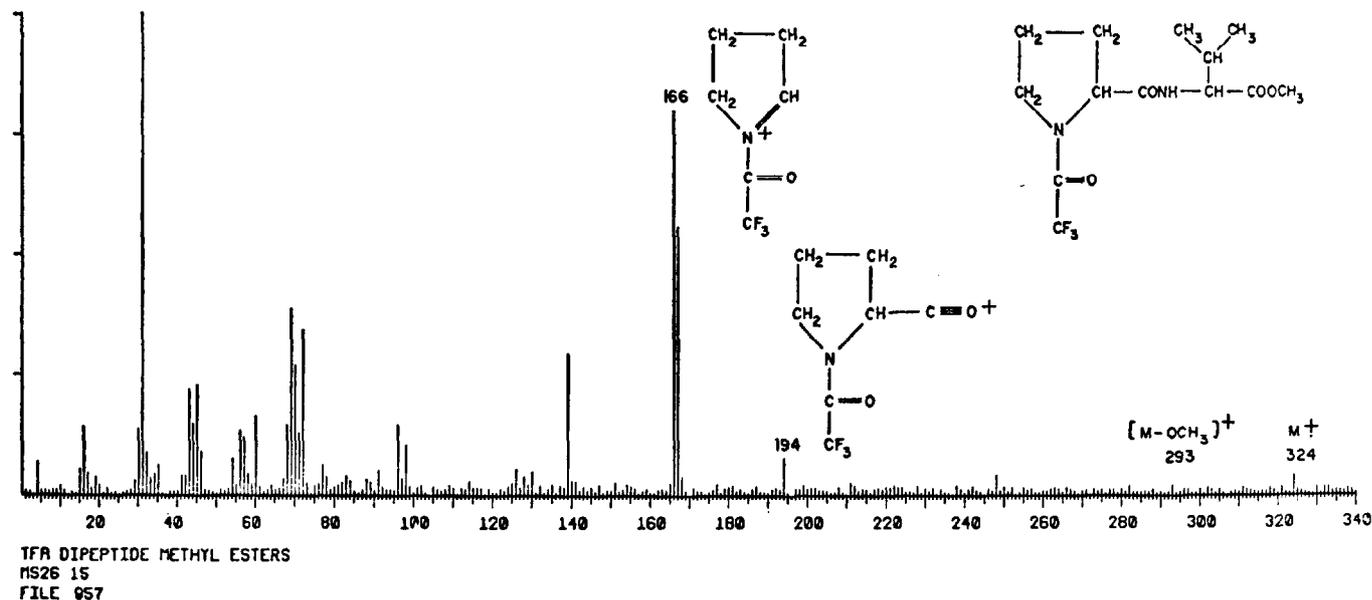


Figure 7. The on-line, computer plotted spectrum

mass values (peaks at 0.5 integer position), wide metastable peaks, or the value of mass defects. It also might be expected that mass defect deviations, especially at high m/e values, would cause difficulty. In practice there have not been any problems attributed to this latter case, the high m/e defect. The resolution and other operational factors have masked this inherent difficulty. If operational use should be hampered by this problem, we have proposed techniques of concurrent calibration on the unknown peaks themselves.

For example, the major peaks in the unknown compound, identified to their nominal integer value (less mass defects), would be used to calibrate the N table. After a programmed computer decision to explore a peak area, similar to those illustrated in Figure 3, the computer would direct the "scan" to that area. Data acquisition time would be about 100 msec for 10 data points over the peak profile. Centroid identification and recalibration might be another 100 msec. If this were repeated for 8 or 10 key peaks, total recalibration would be in the order of 2 sec. This technique could also be employed to verify the linearity above the last reference peak (614 with our system) or below the first. (We have paid little attention to m/e below 12 or 18.)

As a final minimal operating mode, the scan could be programmed to simulate any present mode of time-based scan and data collection, with comparable attributes of performance. As an example, doubly ionized masses or metastable peaks might be identified and measured. Of course the MS used must have sufficient resolution and/or sensitivity, also the total spectrum acquisition time would be increased. Such changes of operational mode would be by program only; hence they could be established or removed in milliseconds with no hardware or switch changes of any nature.

RESULTS

In a typical experiment a mixture of TFA-dipeptide methyl esters (22) was injected into the GLC-MS system, and 130 complete mass spectra were collected and recorded by the computer. Eighty-one of the individual spectra were summed to obtain the total ion plot (Figure 5) (19). This may be compared with the GLC flame ionization chart record on the top. The mass spectral output from peak "C," for example, in the

(22) B. Halpern and J. W. Westley, *Biochem. Biophys. Res. Commun.*, **19**, 361 (1965).

region of scans 955 to 960, was then abstracted by the TYPE routine and the results are shown in Figure 6. This data shows that Peak C is homogeneous and that a satisfactory normalized plot can be obtained from scan No. 957. Figure 7 is the plot as produced in the laboratory. The background in the mass spectrum is primarily due to the GLC column bleed. Only the chemical notation was added by hand.

The taking of spectra, in this computer-compatible form, opens the way to many types of further processing: searching through each stored spectrum to pick out the amplitude at a specified mass position and plot this data to show the variation of the ion current of a specific m/e with time; check the homogeneity of each GLC peak; resolve the mass spectra of simple mixtures into those of the individual components; and subtract background signal from a mass spectrum.

The system is also well suited to the recording of spectra from solid samples introduced directly into the ion source. Because of the fast scanning speed and the recording of many spectra, there is no need to establish a constant vapor pressure in the mass spectrometer and any contamination or decomposition of the sample is readily detected.

CONCLUSION

It is feasible to build a limited purpose computer and a quadrupole MS as one unit, forming a compact instrument with features similar to the system presented in this report. In addition, some of the software could be embodied in hardware circuits. Such units would be more costly than those using commercial general purpose computers, but could be uniquely well adapted to space or other special physical environments. Alternatively, the system can be interfaced with a larger time-shared computer.

RECEIVED for review March 23, 1970. Accepted June 15, 1970. This research was aided by grants from the National Institutes of Health, Public Health Service, AM 12797-01 and FR-00311-04. The LINC computer was provided under NIH grant FR-00151-01. The work is a part of a program of instrumentation research oriented toward automated planetary experiments, supported by the National Aeronautics and Space Administration, research grant NGR-05-02-004. Principal investigator, Joshua Lederberg; program director, Elliott C. Levinthal.

P-7;

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER	
		FROM 1/1/74	THROUGH 12/31/74		
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT	AMOUNT REQUESTED <i>(Omit cents)</i>		
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION	%/HRS.	TOTAL		
Lederberg, J.	Principal Investigator or Program Director	20	SCREENING FOR INBORN ERRORS OF METABOLISM		
Kretchmer, N.	Prof. of Peds.	10			
Cann, H.	Assoc. Prof. of Peds.	10			
Duffield, A.	Research Associate	100			
Pereira, W.	Research Associate	100			
Summons, R.	Post.Doc. Fellow	100			
Rindfleisch, T.	Research Associate	20			
Tucker, R.	Computer Programmer	75			
Veizades, N.	Research Engineer	33			
Steed, E.	Research Engineer	33			
Pearson, D.	Elect. Tech.	40			
Wegmann, A.	Senior Res. Assist.	10			
Open - Genetics	Grad. Res. Assist.	50			
Open - Genetics (continued)	Grad. Res. Assist.	50			
TOTAL →			\$ 157,208		
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$ -		
3. EQUIPMENT <i>(Itemize)</i> see attached list			\$ 110,400*		
4. SUPPLIES See attached list			\$ 16,200		
5. STAFF TRAVEL <i>(See Instructions)</i>	a. DOMESTIC 2 East Coast (1000), 1 Mid-west (300), 1 West Coast		\$ 1,500		
	b. FOREIGN (200)		\$		
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>			\$		
7. ALTERATIONS AND RENOVATIONS Relocate equip., power, etc. for GC/MS Data System			\$ 1,800		
8. OTHER EXPENSES <i>(Itemize per instructions)</i> Mini-computer maintenance \$6,000 Freight on capital equipment \$ 500 Office supplies, telephone, repro., postage, publication costs, etc. \$2,600			\$ 9,100		
9. Subtotal - Items 1 thru 8 →			\$296,208		
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>				
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$	
		POSTDOCTORAL	No. Proposed _____	\$	
		OTHER <i>(Specify)</i>	No. Proposed _____	\$	
	DEPENDENCY ALLOWANCE			\$	
TOTAL \$TIPEND EXPENSES →			\$		
b. TUITION AND FEES			\$		
c. TRAINEE TRAVEL <i>(Describe)</i>			\$		
11. Subtotal - Trainee Expenses →			\$		
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i> →			\$296,208		

continued

1. PERSONNEL	TITLE	% TIME
Wyche, M.	Lab. Tech.	100
Boswell, M.	Lab. Tech.	100
Jamtgaard, R.	Administrator	15
Allen, M.	Secretary - Genetics	25
Murray, R.	Secretary - Peds.	25
Harlow, W.	Machinist	10
Open - Genetics	Lab. Glasswasher	25

3. EQUIPMENT

4. Column Gas Chromatograph		\$14,400*
Mini-Computer System:		
PDP-11/45 (8k memory)	20,700	
Memory (16k)	5,200	
Floating Point Hdw.	5,200	
Prog. Clock	600	
Bootstrap Loader	600	
Industry Tape	10,500	
Disk System	11,700	
Lab Interface Units	4,600	
Interproc. Comm.	800	
	<u>59,900</u>	59,900*
Amino Acid Analyzer		32,500*
Event Counter		2,700*
Digital Voltmeter		900*
	Total Equipment	<u>\$110,400*</u>

4. SUPPLIES

Chemicals, glassware, and lab apparatus	\$5,000	
GC supplies (columns, phases, etc.)	1,100	
Dry ice and liquid nitrogen	500	
Data recording media (GC/MS, Calcomp, etc.)	1,800	
Mini-computer supplies (start-up & continuing)	1,600	
Mass spectrometer repair parts & supplies	2,300	
Electronic parts & supplies	1,900	
Amino Acid Analyzer supplies	2,000	
	<u>\$16,200</u>	
	Total Supplies	\$16,200

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	157,208	168,338	215,892	230,758	246,632		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>							
EQUIPMENT	110,400*	22,500*	17,600*	5,300*	6,000*		
SUPPLIES	16,200	16,900	20,200	21,400	22,800		
TRAVEL	DOMESTIC	1,500	1,800	1,900	1,900	2,000	
	FOREIGN						
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS	1,800						
OTHER EXPENSES	9,100	11,500	12,000	12,700	13,500		
TOTAL DIRECT COSTS	296,208	221,038	267,592	272,058	290,932		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Enter on Page 1, Item 4) →					\$ 1,347,828		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget Explanation attached

BUDGET EXPLANATION

The early phases of the present study have been funded by NASA but that agency is rapidly severing its involvement in biological projects. It has already severely reduced its fiscal support of our program in anticipation of total withdrawal within one or a few years. We have started to reduce laboratory staff in response to those cutbacks and will not be able to maintain this laboratory without new funding (like the present proposal). Since we cannot predict the level of funding we will receive for each of these proposals, we simply note the overlap and will negotiate a suitable joint effort for system development and operation, depending on funds available.

The following is a detailed explanation of the budget for this subprogram. This budget covers only the indicated segment, with an indicated allocation for the time of a number of participants who also appear in other segments. These allocations are integrated into the overall proposal budget summarized later (see Table of Contents for location).

It should be noted that support for Professor Lederberg's time is shown at 20%. This includes not only his participation in this subproject but also his role in overall program direction as Principal Investigator.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

YEAR #1 (Metabolic Screening only)

PERSONNEL: Support is requested for 10% of Professor Cann in Pediatrics (Professor Kretchmer will devote 10% of his effort without grant salary support) for clinical and diagnostic inputs to the program. Drs. Duffield, Periera, Summons, and Ms. Wyche are responsible for the derivatization and chemical analysis of urine samples including GC/MS operations. Support for an operator (Ms. Boswell) for the prescreening amino acid analyzer is included. Mr. Steed will maintain the glasswork aspects of the GC/MS system. Messers Rindfleisch and Tucker will be responsible for system design and software implementation respectively and Mr. Veizades, supported by Mr. Pearson, will design the electronics. Ms. Wegmann operates the GC-High Resolution Mass Spectrometer in the Department of Chemistry and will be available to run high resolution mass spectra on critically important samples. Other part-time personnel (machinist, secretaries, and administrative support) act in necessary supporting roles including assisting with liaison work with collaborating physicians at Stanford and elsewhere.

CAPITAL EQUIPMENT: We request a PDP-11/45 mini-computer system as an extension of the data system for our low resolution mass spectrometer. This system will initially have 24K words of memory and use a 1.2M word disk system for monitor, program, and spectral data storage. Removable data storage for raw data and archival spectrum recording is provided by the requested industry compatible magnetic tapes. The terminal, loader, clock, instrument interface hardware, etc. complete the system. Funds to maintain the computer system under manufacturer contract are included under "OTHER." A detailed computer equipment list is included in the "First 12 month budget detail."

We also request a 4 column gas chromatograph and an amino acid analyzer which will increase our urine prescreening capacity thereby alleviating the bottleneck at the mass spectrometer. The latter instrument is particularly pertinent to the larger scale prescreening of samples from the Kaiser Hospital, the Santa Clara Valley Medical Center, the Children's Hospital at Stanford, and the LA County Hospital. The amino acid analyzer will also allow reliable continued access to screening facilities for routine clinical studies during times when the GC/MS may be tied up for more sophisticated studies, or may be down for maintenance, repair, or re-engineering. The reliability offered by this back-up will help resolve the problem faced by any clinician when he attempts to investigate new techniques while committed to offering the highest standard of care. Once an analytical technique is coupled to patient care, it cannot ethically be disengaged - a priority that puts great strains on any system also intended for further research. In the present context, once a metabolite has been identified, it may be efficient to monitor its level, in repetitive samples, by techniques like the amino acid analyzer.

We have access to both low resolution and high resolution mass spectrometers (physically located in the Genetics and Chemistry Departments respectively) and do not request augmentation of this costly hardware for the present proposal.

SUPPLIES: Supplies are requested for sample preparation and equipment operation. These include chemicals, glassware, laboratory apparatus, GC supplies, amino acid analyzer supplies, dry ice and liquid nitrogen, recorder paper for the GC/MS instruments and Calcomp plotters, and computer operation supplies. In addition, we request supplies and parts for the maintenance (by our own hardware personnel) of the GC/MS system and electronic equipment as well as for the implementation of necessary data system interface hardware.

TRAVEL: Travel costs are estimated at round trip expenses for two professional meetings on the east coast and 1 trip each to the mid-west and west coast.

ALTERATIONS AND REMOVATIONS: We will need to modify the electrical service and relocate some equipment in order to install the new data system and gas chromatograph and to allow for future equipment we are requesting. This estimate is based on a similar modification just made in another laboratory.

OTHER EXPENSES: Office supplies, telephone service, postage, publication costs, etc. are requested based on operating experience in our laboratory. Computer maintenance and equipment freight are estimated for the proposed data system augmentations.

YEAR #2

PERSONNEL: Personnel are unchanged in year 2.

CAPITAL EQUIPMENT: As the data system adaptation reaches completion after year 1, we request additional machine features to facilitate operational use simultaneously for GC prescreening and for MS analyses. These include a block of fast memory (1K words) to speed up the machine, an additional disk drive for larger volume on-line storage requirements and a high speed display device for system status and processing result feedback. Appropriate equipment maintenance increases are included for the expanded data system.

SUPPLIES, TRAVEL, AND OTHER: These items are increased by 6% to reflect inflation and the needs of increased throughput of urine screening.

YEAR #3

PERSONNEL: We anticipate an increased volume of samples to be processed by the third year as our baseline studies progress and more extensive clinical screening is undertaken. This implies the need for additional support both at the clinical and laboratory level. For this reason we have added 50% time of an Assistant Professor of Pediatrics and 100% time for a laboratory technician for sample preparation and equipment operation to the budget.

CAPITAL EQUIPMENT: An additional prescreening GC is requested to allow larger population analyses along with the appropriate computer interface equipment.

SUPPLIES, TRAVEL, AND OTHER: The budget for supplies and equipment maintenance are increased consistent with personnel and equipment additions. Also provision is made for inflation and longer daily operation.

YEAR #4

PERSONNEL: Personnel remain unchanged in year 4.

CAPITAL EQUIPMENT: We request a system for the vacuum distillation of aqueous samples for specimen preparation and derivatization as well as miscellaneous test equipment which will require replacing.

SUPPLIES, TRAVEL, AND OTHER: Increases are requested to cover expected inflation.

YEAR #5

CAPITAL EQUIPMENT: A small provision is made for replacement test equipment.

SUPPLIES, TRAVEL, AND OTHER: Increases similar to year 4 are requested.

SECTION III

Maternal Blood Stream - Another Source of Fetal
Tissue for Pre-Natal Diagnosis of Genetic
Disorders

Drs. Herzenberg and Cann

The Maternal Bloodstream - Another Source of Fetal Tissue for Prenatal Diagnosis of Genetic Disorders

Dr. L. A. Herzenberg, Principal Investigator
Dr. H. Cann, Associate Investigator

A. INTRODUCTION

A.1 Objectives

We propose to study methods of labelling, detecting and isolating fetal lymphocytes which pass into the maternal circulation during gestation. Further data on the time, extent and conditions of occurrence of these cells during normal human pregnancy could lead to non-invasive methods for prenatal diagnosis of genetic disease.

A.2 Background and Rationale

Fetal lymphocytes have been reported to be present in the mother's blood in the majority of pregnancies; they may be present in the maternal circulation in all pregnancies. The frequencies of fetal cells found, that is the percentage of fetal lymphocytes in the pregnant woman's blood stream, have ranged from 0.05% to as high as 1.5% in two studies. In one, by Walknowska, Conte and Grumbach (1), the presence of 5 small acrocentric chromosomes was sought in lymphocytes obtained from 10 milliliters of maternal blood, stimulated by phytohemagglutinin (PHA) and examined at metaphase. From one to three metaphases out of many hundreds, suggested a male fetus in 21 pregnancies. This diagnosis was checked at birth and 19 males were correctly predicted. Three of these mothers were studied at 20 weeks or less of gestation. In the second study (2), quinacrine fluorescent-staining Y bodies were looked for in interphase lymphocytes obtained from blood (20 milliliter specimens) of women in the second and third trimesters of pregnancy. Here eleven male infants were predicted and seven were verified at birth. Since the method used by Walknowska et al. is relatively insensitive, we suggest that fetal lymphocytes are present in the maternal bloodstream in frequencies of about 0.1% or more in all pregnancies at least from 14 weeks of gestation on.

Fetal lymphocytes possess HL-A antigens perhaps as early as 12 weeks of gestation (3). The enormous amount of polymorphism noted in Caucasian populations (4) is such that most individuals will be heterozygous at one or both HL-A sub-loci and the likelihood of mates differing at one or both sub-loci is high. These facts suggest that fetal lymphocytes may be separated from the maternal bloodstream by taking advantage of the HL-A differences existing between parents. A cytotoxic antibody directed toward the maternal HL-A antigen which the fetal lymphocytes do not carry, will, in the presence of complement, lyse the maternal leukocytes sparing those of the fetus. Or,

indirect labeling of the fetal lymphocytes coated with antibodies to the paternal HL-A type with fluorescein conjugated anti-human globulin will render these fluorescent in a sea of non-fluorescing maternal cells.

The fluorescent cell-separator perfected by engineers working under the direction of one of us (Herzenberg) can separate fluorescent from non-fluorescent cells with high efficiency, viability and yield (Figure 1; also reference 5). It can detect fluorescent-labeled cells present in frequencies lower than 10^{*-5} and can separate and thus enrich by a factor of 10^{*3} . In model experiments with human erythrocytes, Rh+ cells stained with anti-D and fluorescein-conjugated anti-human immunoglobulins were detected about one time in two at a frequency of one positive cell per 10^{*5} Rh negative cells. Post-partum blood samples of Rh- mothers who just gave birth to an Rh+ child were found to have from 1/163,000 to 1/5,000 Rh+ cells indicating fetal blood leakage, probably at birth, of from 20 to 680 microliters (6). Thus, it should be quite feasible to enrich fetal lymphocytes from the reported frequencies of 10^{*-3} to close to purity after the fetal cells are stained by immunofluorescence for paternal HL-A antigens.

We recognize the potential of the cell separator to separate fetal red blood cells from the maternal bloodstream on the basis of heritable erythrocyte antigens. Such methodology could lead to the development of prenatal diagnostic tests for sickle cell anemia, possibly thalassemia, and other hereditary disorders of the erythrocyte. We have decided, however, to emphasize isolation of fetal lymphocytes in this research project because of the reports (1,2) suggesting that these cells are detectably present in the maternal bloodstream in most, if not all, pregnancies. The literature suggests that fetal red blood cells are detectable in the maternal circulation in 5-10% of pregnancies at 20 weeks of gestation or less (7,8). Whether the application of the cell separator to detection and separation of fetal red blood cells will improve these figures, is a question we have been discussing. We believe that more data on the frequency of mothers showing fetal red blood cells in their circulations and the frequency of the cells themselves at various times in pregnancy are needed, and the cell separator could be applied to this research question. At present, our estimates of the amount of fetal red blood cells we can isolate, based on the performance of the cell separator and on the amount of fetal blood reported in the maternal circulation, indicate that our yield will be too low (perhaps 100 times too low) to permit diagnostic studies based on incorporation of radioactive label into globin synthesized by fetal cells (9,10). For these reasons too, we are persuaded to work with fetal lymphocytes. However, since we developed and used the methodology to detect Rh- fetal cells in post partum Rh+ maternal blood specimens (6), we are planning preliminary experiments designed to answer questions as to amount, frequency, and requirements, raised above. We are collaborating with Dr. Herbert Schwartz, Professor of Pediatrics and Chief of the Pediatric Hematology Service at Stanford, in this preliminary

work. Dr. Schwartz will review the morphology of and determine whether globin synthesis can be measured in cells separated from the maternal circulation. Our preliminary work will be aimed at ways to increase yield and/or decrease amount of fetal blood required for prenatal detection tests. At this time, we are not asking for budgetary support in this area, although we may, should preliminary work show promise.

The remainder of this proposal will deal only with the separation of fetal lymphocytes from the maternal circulation.

B. SPECIFIC AIMS

We propose to use heritable surface antigens as a basis for separating fetal lymphocytes from the maternal bloodstream during gestation. These separated cells will be used to diagnose various genetic abnormalities in the fetus.

C. METHODS OF PROCEDURE

C.1 Immunoselection of Lymphocytes

(a) POSITIVE IMMUNOSELECTION WITH THE FLUORESCENT CELL SEPARATOR. Specific fluorescent staining for a variety of HL-A antigens will be developed. In most cases, we will use the indirect procedure, i.e. anti HL-A whole serum followed, after washing, by fluorescein or rhodamine conjugated anti-human immunoglobulin (F or R anti-Ig). In pilot studies for this project, we have already obtained staining sufficiently bright to achieve excellent cell separation with a polyspecific and two specific HL-A antisera. In order to decrease non-specific staining (of lymphocytes lacking the antigens to which the antibodies are directed) we have had to remove aggregated proteins of antiserum by centrifugation at 100,000 g for 2-3 hours.

Lymphocytes are prepared by ficoll-Isopaque isopycnic centrifugation (11) followed by filtration through nylon wool to remove monocytes and many B-lymphocytes. Since these latter have surface immunoglobins, they stain with the fluorescent reagents, although much more dimly than the HL-A stained cells. With these virtually pure lymphocyte population, of which greater than 90% are thymus-derived (T) lymphocytes, we have obtained bright speckled fluorescence by staining at 0 degrees C. When we have passed positive and negative (stained for a particular HL-A type) cells separately through the cell separator, the fluorescence intensity distribution has shown two clear populations with very little overlap (Figure 2). Thus, good separation can be obtained.

Initially, reconstruction experiments will be performed using various mixtures of cells positive and negative for an HL-A antigen or antigens. In addition to numerical evidence (from the fluorescence intensity distribution) for successful separation, we will use male and female cells so that sex chromatin, karyotyping and quinacrine staining for Y bodies (2) can be used as confirmatory markers.

In isolations from maternal blood, antibodies directed to paternal and not maternal antigens will be used followed by F anti-Ig.

(b) **NEGATIVE IMMUNOSELECTION WITH COMPLEMENT MEDIATED LYSIS.** As an alternate procedure to or a step preliminary to fluorescent cell separation, (maternal) cells with particular HL-A antigens will be killed by incubation with antiserum and complement. Antisera directed against maternal antigens not shared by the father will be used when possible. With separate aliquots of lymphocytes, antisera negative for one or the other set of paternal antigens will be used.

C.2 Fetal Lymphocyte Antigens as Alternates to HL-A for Immunoselection with the Fluorescent Cell Separator

Several fetal antigens have been described (12) but, so far, none of lymphocytes. If an antigen could be found to be present on early fetal lymphocytes but absent, or in greatly diminished amount, on adult lymphocytes, it would be considerably more convenient to use than HL-A antigens. There would be no problem of typing the father and having to select for each of the paternal haplotypes.

We will attempt to find a fetal antigen by direct immunization of other species with lymphocytes from aborted fetuses. Any antisera obtained will be absorbed with adult cells, including lymphocytes, to render it specific. Of course, we cannot predict in advance if this will succeed. Another means to look for fetal lymphocyte antigens is to use antisera raised against leukemias. Tumors often have fetal or embryonic antigens; whether leukemias do is unknown. We will test for them with fetal and normal adult lymphocytes.

C.3 Mitogens Preferentially Stimulating Fetal Cells.

It is possible that some mitogens will be relatively more stimulatory to fetal than adult lymphocytes. If so, this would decrease the importance of maternal contamination of separated fetal cells. We will screen all the available lectins including PHA, PWM (pokeweed mitogen), concanavalin A, wheat germ agglutinin, etc. for mitogenic activity with cells from aborted fetuses of several ages and cells from adults. One precedent for differential mitogenesis is PHA which stimulates fetal mouse thymocytes 10 times more than adult mouse lymphocytes (13). Evidence of differential response to PHA and PWM by lymphoid cells of human newborns (cord blood) and adults suggests that this approach may be feasible (14).

C.4 Lymphocyte Culture and Karyotyping.

Separated lymphocytes will be grown in micro-culture plates with phytohemagglutinin (PHA) for 48 hours, colcemid added and chromosome spreads made 16-20 hours later. In preliminary experiments we have obtained high percentages of metaphases with

50,000 cells cultured in 250 microliters of medium. Prior staining with anti HL-A does not seriously decrease (less than 2-fold) the mitotic index. We have been successful in obtaining high quality chromosome spreads by standard techniques with these small numbers of cells in micro-cultures. We believe we could culture (in 10 microliters medium) and spread as few as 1,000 lymphocytes. However, that is yet to be tried.

To check our ability to separate, grow and karyotype fetal lymphocytes, we will predict the sex of the fetuses and compare with the sex observed at birth.

C.5 Cell Separations

Fluorescent cells will be separated in the fluorescent cell sorter we have built (see Figure 1). Cells suspended in medium pass in single file in a stream past 2 laser light sources and 2 photodetectors. One laser and detector observes cell fluorescence and the other light scatter which is a measure of cell volume. When a cell with the desired fluorescence level and of the right volume is detected, a charge is applied to the stream with a delay of about 100 microseconds. The stream is broken into 40,000 uniform droplets per second downstream of the observing point. Charges are applied when droplets containing the desired cells are just being formed so they become charged. They are then deflected by constantly charged deflecting plates into collecting tubes.

The number of cells processed per unit time is generally inversely related to the enrichment obtained. With a frequency of desired cells of $10^{(-3)}$, 10^{+4} cells per second can be processed to obtain about 100% purity of wanted cells. A yield of at least 20,000 desired cells per hour should be obtained. This is certainly enough to culture and karyotype. If the frequency of fetal lymphocytes in the maternal bloodstream is 10-fold lower, i.e. $10^{(-4)}$, then only 2,000 cells per hour would be obtained. This is probably a marginal number for culture. Furthermore, there would probably be prohibitable losses with so few cells. However, there is no need to seek high purity. When looking for male karyotypes, or later for gross chromosome abnormalities, only a few successfully spread cells of the selected type are needed. If we predict 5% purity, we need to screen about 100 cells to be quite certain of seeing a few metaphases from the selected cells. Thus, preliminary analysis of maternal samples will be used to determine the amount of cells to be put through the cell sorter.

With the $10^{(-4)}$ frequency, which is at least 10 times lower than the published estimates, $2 \times 10^{+7}$ lymphocytes from maternal blood will be processed per hour. This approximately represents the number in 10 milliliters of blood. For a routine, non-objectionable procedure, we feel this (or perhaps 20 milliliters) is about the limiting amount of blood which could be used. Thus, for screening purposes, the minimum fetal lymphocyte frequency is probably slightly less than $10^{(-4)}$. We will

establish what the real frequencies are in a reasonable number of pregnant women at various stages of gestation. If the published values are correct, we will not be limited by fetal cell frequency. (For high risk pregnancies, it may be that even with a frequency of less than 10^{*-4} , it would be preferable to do the antenatal cytogenetic analysis with 50-100 milliliters of blood rather than by amniocentesis).

C.6 Strategy (and Summary)

(a) HL-A specific staining by immunofluorescence will be developed. This is presently in progress.

(b) Preliminary enrichment by cytotoxicity with HL-A antisera and complement will be assessed.

(c) Separation from artificially mixed populations will be carried out using sex markers to confirm.

(d) Fetal lymphocyte frequencies will be determined at various stages of gestation.

(e) Fetal lymphocyte antigens will be sought.

(f) Selective fetal mitogens will be sought.

(g) If the frequency in late first trimester or early second trimester pregnancies is about 10^{*-4} , separations, culture and karyotyping will be done. Accuracy of prenatal sex prediction will be used to confirm separations.

Time Table: Development of the method with artificial mixtures and maternal blood will take between one and two years. Assessment of frequency of fetal lymphocytes in maternal blood as a function of gestational time will take about six months.

Subsequent steps involve developing enough reagents for a significant trial at detecting chromosome abnormalities using high risk pregnancies. This stage will also require setting up a cooperative effort involving patients at Stanford and other nearby institutions (e.g. the University of California Medical Center, San Francisco; Dr. C.J. Epstein). This will involve comparing results from the cell separator technique with those from amniocentesis. We anticipate applying for supplementary funds after 3 years if this pilot project is successful.

D. SIGNIFICANCE

A routine method of antenatal diagnosis using 10-20 milliliters of maternal blood would permit mass screening for genetic disorders, especially those involving chromosomal aberrations. The methods we propose could result in avoiding the use of amniocentesis even in high-risk pregnancies (amniocentesis would be difficult to apply for mass screening).

The cost of separation and karyotyping could be brought into the range of a few dollars per test with the development of semi-automated micro-cultures and computer assisted karyotyping (Dr. C.J. Epstein, personal communication). The cost of the 0.5-1% of congenital chromosome abnormalities among births is far higher than the cost of such screening. It is impossible to assign a uniformly acceptable cost in total human terms, but the actual costs to the family and/or society must be in excess of 100,000 dollars for each infant with Down's syndrome born. Screening to detect an extra chromosome 21 would yield 1 case in 600 at a cost of probably from 1,000-10,000 dollars. Thus, the cost would likely be justified even for only this one condition.

Further work could permit biochemical and particularly cytochemical tests (15) for inherited disease to be performed on the separated fetal lymphocytes either before or after culture.

We wish to stress that the use of maternal blood rather than amniotic fluid samples provides a non-invasive method of antenatal diagnosis. This methodology can eliminate the risks to fetus and mother which are encountered in amniocentesis.

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TITLES OF FIGURES

Figure 1. Diagram of fluorescent cell separator.

Figure 2. Demonstration of almost complete separation of HL-A stained and unstained cells.

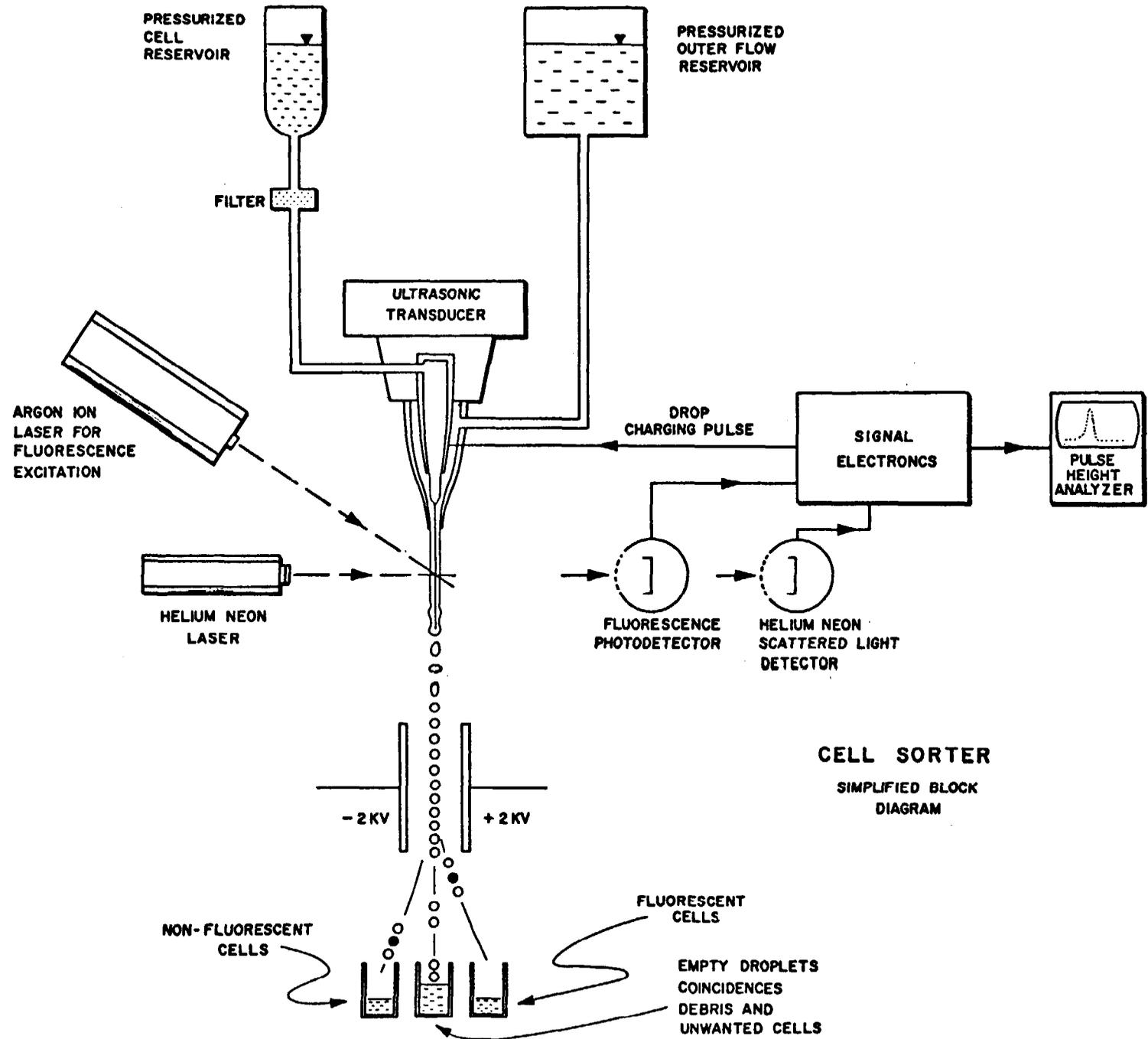


FIGURE 1

P-95

Frequency Distribution

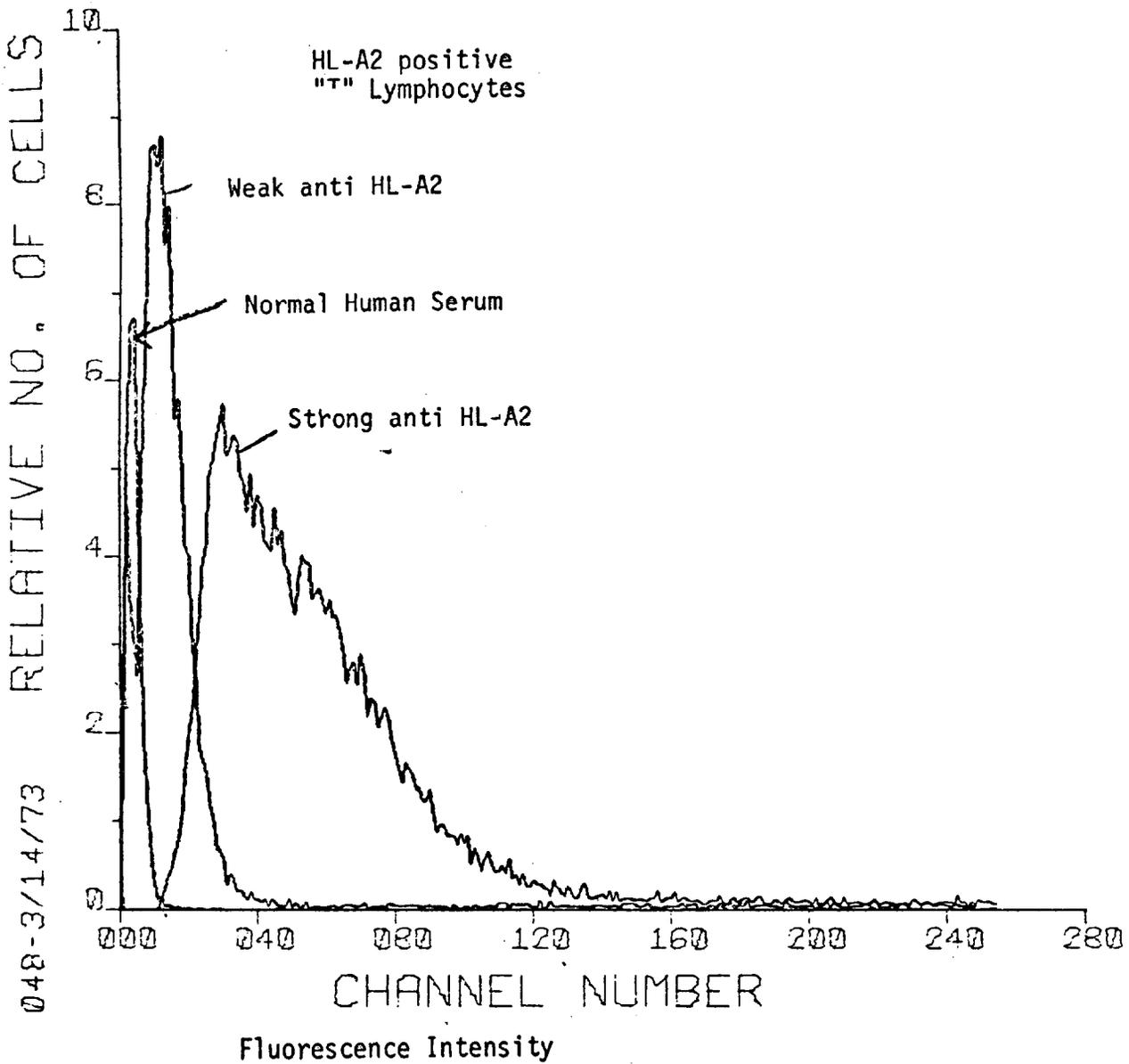


FIGURE 2

SUBSTITUTE DETAILED BUDGET FOR FIRST 12-MONTH PERIOD		PERIOD COVERED		GRANT NUMBER
		FROM 1/1/74	THROUGH 12/31/74	
1. PERSONNEL <i>(List all personnel engaged on project)</i>		TIME OR EFFORT %/HRS.	AMOUNT REQUESTED <i>(Omit cents)</i>	
NAME <i>(Last, first, initial)</i>	TITLE OF POSITION		TOTAL	
Herzenberg, Leonard	Principal Investigator or Program Director	10%	FETAL CELLS IN MATERNAL CIRCULATION	
Cann, H.	Assoc. Prof. Peds.	10%		
Open - Pediatrics	Research Associate	100%		
Hulett, H.	Research Associate	20%		
Waters, Ruth	Research Assistant	100%		
Sakaguchi, S.	Research Assistant	50%		
Open - Genetics	Engineering Tech.	50%		
Meyering, P.	Secretary	10%		
TOTAL →			\$ 54,782	
2. CONSULTANT COSTS <i>(Include Fees and Travel)</i>			\$	
3. EQUIPMENT <i>(Itemize)</i>				
	Carbon Dioxide Incubator	\$2,500		
	Laminar Flow Hood	1,000		
	Fluorescence Microscope	9,000		
			\$ 12,500 *	
4. SUPPLIES				
Tissue culture supplies, (filters, trays, etc.), dishes, media, pipets, sera, etc.			\$ 7,000	
5. STAFF TRAVEL <i>(See Instructions)</i>				
a. DOMESTIC	1 East Coast meeting		\$ 500	
b. FOREIGN			\$	
6. PATIENT COSTS <i>(Separate Inpatient and Outpatient)</i>			\$	
7. ALTERATIONS AND RENOVATIONS			\$	
8. OTHER EXPENSES <i>(Itemize per instructions)</i>				
Office supplies, telephone, repro., postage, publication costs, etc.			\$ 1,000	
9. Subtotal - Items 1 thru 8 →			\$ 75,782	
FOR TRAINING GRANTS ONLY	10. TRAINEE EXPENSES <i>(See Instructions)</i>			
	a. STIPENDS	PREDOCTORAL	No. Proposed _____	\$
		POSTDOCTORAL	No. Proposed _____	\$
		OTHER <i>(Specify)</i>	No. Proposed _____	\$
		DEPENDENCY ALLOWANCE		\$
	TOTAL \$TIPEND EXPENSES →			\$
b. TUITION AND FEES			\$	
c. TRAINEE TRAVEL <i>(Describe)</i>			\$	
11. Subtotal - Trainee Expenses →			\$	
12. TOTAL DIRECT COST <i>(Add Subtotals, Items 9 and 11, and enter on Page 1)</i>			\$ 75,782	

**BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)**

DESCRIPTION	1ST PERIOD (SAME AS DE- TAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED <i>(This application only)</i>					
		2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS	54,782	58,659	62,704	67,021	71,629		
CONSULTANT COSTS <i>(Include fees, travel, etc.)</i>							
EQUIPMENT	12,500*						
SUPPLIES	7,000	7,500	7,900	8,400	8,900		
TRAVEL	DOMESTIC	500	600	600	600	700	
	FOREIGN						
PATIENT COSTS							
ALTERATIONS AND RENOVATIONS							
OTHER EXPENSES	1,000	1,100	1,200	1,200	1,300		
TOTAL DIRECT COSTS	75,782	67,859	72,404	77,221	82,529		
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD <i>(Enter on Page 1, Item 4)</i> →					\$ 375,795		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Budget explanation attached.

BUDGET EXPLANATION

The budget covers 10% each of Professor Herzenberg's and Professor Cann's time in relation to this subproject. A full time Research Associate is required to obtain, prepare, and fluorescent label the lymphocytes for separation and then to culture and perform cytogenetic studies on the separated cells. The part time (20%) Research Associate is to be responsible for the actual separations. Two Research Assistants, one part-time (50%) for cytogenetic studies and one full-time for serological studies, as well as a part-time (50%) cell separator operator are required to assist in these procedures. Support is also budgetted for 10% of Professor Herzenberg's secretary.

Salaries are increased at a rate of 6% per year to cover merit and cost of living increases. Staff benefits are applied based on the following University projections: 17%, 9/73-8/74; 18.3%, 9/74-8/75; 19.3%, 9/75-8/76; 20.3%, 9/76-8/77; 21.3%, 9/77-8/78; and 22.3%, 9/78-8/79.

The laminar flow hood is essential for aseptic culturing and protection of personnel from possible infectious agents in human blood. The carbon dioxide incubator is required for culturing lymphocytes. The fluorescent microscope will be used to detect interphase fluorescent Y bodies and the Y chromosome at metaphase. In the later stages of this study, the fluorescence microscope will be used to study the karyotypes of lymphocytes of fetuses with chromosomal aberrations.

The budget for this project represents the MINIMUM ADDED COSTS to Dr. Herzenberg's and Dr. Cann's other programs (cell separator project, in particular) required for this work to be performed.

SECTION IV

Polymorphic Genetic Markers in Amniotic Fluid

Drs. Cann and Tsuboi