FINAL REPORT

CYTOTOXICITY ASSAY AUTOMATION

Contract Period Covering June 17, 1969 to October 31, 1971
For
National Institute of Allergy and Infectious Diseases
Transplantation Immunology Branch
National Institutes of Health
Contract 69 2064

Instrumentation Research Laboratory, Department of Genetics
Stanford University School of Medicine
Stanford, California 94305
FINAL REPORT

TO THE

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

TRANSPLANTATION IMMUNOLOGY BRANCH

NATIONAL INSTITUTE OF HEALTH

Contract Number NIH 69-2064

For the Period June 17, 1969 to October 31, 1971

"CYTOTOXICITY ASSAY AUTOMATION"

Instrumentation Research Laboratory, Department of Genetics

Stanford University School of Medicine

Stanford, California

Elliott C. Levinthal
Principal Investigator

Rose O. Payne
Co-Principal Investigator
INTRODUCTION

Since June 17, 1969 the Department of Genetics of the Stanford University Medical School has been working under contract NIH 69-2064 on a system designed to automate testing for HLA antigens. The major objectives of the program have been to design and build a laboratory breadboard of such an automated cytotoxicity testing system (ACTS), to test and evaluate the performance of the unit, and to compare its performance with non-automated tissue typing techniques. This final report summarizes the work under this contract. Dr. Walter Bodmer, now at Oxford was the original principal investigator on this program and was directly involved in it until the end of 1970. We have accordingly asked him for his comments on the results of the project which are included as Appendix I.

We decided early in the project that the fluorochromatic cytotoxicity assay developed by Bodmer was most applicable to this work. In this assay cell suspensions are incubated with fluorescein diacetate (FDA). The nonfluorescent FDA passes readily through the cell wall. Inside the cell it is hydrolyzed by intracellular esterases producing free fluorescein. The fluorescein leaks from the cells very slowly. As a result those cells with intact cell membranes show a yellow-green fluorescence when illuminated with blue or long ultra-violet light. If the cells are later incubated with serum and complement, any incompatible serum will cause cell lysis. As soon as the cell wall is ruptured, the fluorescein leaks out quickly and the cell is no longer fluorescent.
This loss of fluorescence is thus an indication of presence of antigen. In the present method the cells are simply observed in a fluorescent microscope after a suitable incubation time. The relative number of fluorescent cells is determined. A positive reaction is evidenced by a low fluorescent cell count. With the ACTS the cell suspension is made to flow past a fluorescence detector and the fluorescent cell count determined electronically. An advantage of this assay is that not all the cells in any sample need be detected. So long as a control sample is run, with a known compatible serum, the counts in each sample can be compared with those in this control.
Description of Instrument

An overall view of the completed ACTS is shown in Figure 1, and a view of the central unit, the delivery system and optical assembly, is shown in Figure 2. The system was designed to pick up samples sequentially from a Terasaki plate containing 60 sample cells, inject them into a coaxial flow system, and examine the fluorescence of each cell in the sample as it flows through filtered light from a mercury arc source.

The entire operation is performed automatically and requires a total of 6 minutes for the entire scan (60 samples).

The instrument can be divided into the following basic parts:

1. Delivery System
2. Optical Assembly
3. Electronics
1. **Delivery System**

The delivery system is an electromechanical assembly designed to deliver each sample sequentially to the optical channel. A Hamilton microsyringe is used to pick up the sample and deliver it in the optical channel. All motions are pneumatically controlled with the exception of the indexing of the Terasaki plate which is done with electrical indexing motors.

Each cycle incorporates the following operations:
1. Slides Down (Injection of syringe into solution).
2. Syringe Piston Down.
3. Syringe Piston Up (Pick up of sample).
4. Syringe Piston Down (Injection of sample back into the plate for mixing purposes).
5. Syringe Piston Up (Pick up of sample).
7. Arm Rotation (Moving of sample containing syringe 90° from the pick-up station to the optical channel station).
8. Slides Down (Insertion of syringe into the optical channel).
9. Syringe Piston Down (Injection of sample into the optical channel).
10. Slides Up (Withdrawal of syringe from optical channel).
12. Arm Return (Moving of empty syringe back to sample station). (During this time an optional wash cycle may be activated to wash out any residue of the previous sample. This cycle is not used at present.)
During steps 7 through 12 of the above sequence the Terasaki plate is moved so as to position the next sample at the sample station. The sequence is achieved using a timing motor operating a set of cams. The cams in turn activate microswitches energizing electrically operated pneumatic valves which direct compressed air to the appropriate air piston for the required motion. In this prototype instrument there are a total of eight adjustable cams, which singly or in combination produce the appropriate timing sequence for the above listed operations of the sample delivering mechanism. Figure 3 shows a timing diagram of the sequence and the cams that are used for each operation. Figure 4 shows the electrical diagram for the indexing of the Terasaki sample plate holder.

There are two modes of operation, manual and automatic. The manual mode enables the operator to move the plate to any one of the sixty sample cavities of the plate. This is done by switching the AUTO-MANUAL switch into the manual position and activating any of the four direction push buttons (+x, -x, +y, -y) until the desired sample holder cavity is under the syringe. Then by depressing the start button the sampling mechanism is energized for one complete cycle. In the automatic position the operation repeats for up to sixty complete cycles depending on the starting coordinates. In any event at the end of the automatic cycle the tray returns to the starting position (X,Y). When the home position switch is energized the cycle stops and the tray returns to its origin (X,Y). The x and y movements are achieved by utilizing two A-C motors.
Figure 3
to form an x-y coordinate moving stage. Each station is identified by microswitches mounted on the stage. (10 microswitches for the ten possible x positions and six microswitches for the y direction.) The pattern of the plate scan is shown in Figure 5.

Alignment of Delivery Systems

Several basic operations must be performed initially in order to assure proper alignment of the delivery system.

First, the travel of the syringe piston must be adjusted in order to assure proper volume pick up. This is achieved by an adjustable collar on the syringe holding assembly which limits the upward travel of the syringe piston. Nominally this has been set for a total pickup of 3.5 microliters with 6 to 7 microliters in the well.

The arm movement must be set so that at the sample pick up position the syringe is directly above the first sample cavity. The plate holder is constructed so as to allow additional adjusting flexibility. The in-out motion provides the adjustment necessary for proper distance setting from the rotational center of the arm while the up and down motion adjusts for the proper elevation of the plate for accurate pick up of the sample. The stop provided for the arm at the Injection station must be adjusted so that the syringe needle falls into the injection cavity. The optical channel holder incorporates in-out and up-down adjustments. These adjustments provide the necessary flexibility in alignment of the holder. Also stops are provided so as
TERASAKI PLATE SCAN PATTERN

Figure 5
to permit removal of the entire optical channel assembly for cleaning purposes and reinserting it without necessitating readjustment.

2. Optical Channel

The optical channel is shown in block diagram form in Figure 6. The injected sample flows through the two capillary orifices of a laminar sheath flow system similar to that described in Merrill et al.\(^{(2)}\). The system operates by vacuum (15 inches Hg gauge) which pulls the solutions into a laminar stream of about 50 μ in diameter which emerges from the second nozzle into a viewing chamber.

The stream is illuminated by a beam from a 100 watt mercury arc source focused by a dark field condenser. The second (outside) nozzle is a 50 μ glass capillary manufactured by Specialty Glass Co., and fed as shown by an external normal saline solution reservoir. The inner nozzle is a 75 μ glass capillary manufactured by the same company. A pool of saline fed by a separate saline reservoir is always present on top of the inner nozzle channel. This keeps air from being drawn into the channel during operation. It also washes off oil deposited on the syringe needle during the pick up of the sample. A dual 30 μ stainless steel mesh filter is used in line with the inner stream in order to prevent clogging of the inner needle by debris that might be picked up and injected by the syringe. The sample containing the stained cells is injected into the inner nozzle channel forming a stream which flows inside the outside sheath through the outer nozzle. Due to the
mechanical configuration and the dynamic forces exerted on the inner stream containing the stained cells it emerges at about 20 μ diameter. When the up-stream pressures are adjusted correctly by relative elevations of the feed containers, the cell sample is depleted within one cycle. Thus mixing of successive samples is minimal and coincidence of detection is reduced. Total fluid flow rate is about 1.5 ml/min.

The optics of the instrument, shown in Figure 7, are mounted on a separate optical table. This optical table can be elevated up and down with a chain drive mechanism. The objective portion of the microscope which also incorporates the detector (phototube) is attached through an x-y stage to the optical platform. The Y-adjustment is used to bring the objective in line with the stream and the X-adjustment is used for focusing. Z-adjustment is achieved by moving the entire optical table with respect to the nozzle assembly.

The 100 watt Hg arc source is mounted on the optical table with adjustments necessary to position it with respect to the back of the condenser.

The condenser (dark field) is mounted on an x-y-z stage for proper adjustment of the illuminating beam. Adjusting the condenser for maximum output signal is difficult and the operator should be familiar with dark field illumination before any attempt is made to make these adjustments.
Figure 7
3. **Electronics**

The electronics incorporated in this instrument are divided into two systems. One includes the necessary electronics required for the mechanical manipulation of the instrument. These are the timer unit with the associated cams which energize the air valves for operating the mechanical movements, and the circuitry for indexing the Terasaki plate. The second system is associated with the detection and retrieval of the electrical signal.

The timer, air valve and control operations have been described briefly above. Figure 4 shows the electrical diagram of the control unit for the Terasaki plate. All of the switching in this unit is done with electromechanical switches (relays, microswitches). This approach was chosen because the high noise generated by the other electromechanical devices used in this instrument meant that the control unit had to be designed with good noise immunity and reliability.

The cycle begins with depressing the START button with the tray at 1A position. The start button energizes relay K8 which is self-holding through contact K7. Cam 8 switch applies a signal through K7, K5, K6, K4, and K2 to X+ relay energizing the X-motor. X-relay is self-holding through X+, K0, K6, and K5. When the tray reaches position 2 relay K0 is energized causing X+ to de-energize due to K0 contact. This sequence is repeated through all 10A positions. When number 10 switch is depressed relay K6 is energized through K2 and K4A. The next cam 8 signal is now directed to Y+ relay and is self-holding through Y+, K4A, K3, K7,
K2A₃ and Switch A. When position B is reached, K2, K2A are energized causing Y+ to stop the rotation of the motor through K2A₃. Now the tray is in 10B position in relation to the syringe. The next cam signal is directed to the X-relay through energized K2₇ relay. This X-relay drives the X-motor in the negative direction. The (X-) relay is self-holding through X₋, K₃, K₆₇ (already de-energized due to K₂₃) and K₅₇. Thus when X₋ switch is activated, K₀₃ causes the X- motion to stop.

Now with successive cam signals the tray moves in the X- direction on Row B. This sequence is continuous up to the 1B position. In this position relay K₅ through K₁₃ and K₃₃ is energized directing the next cam signal through K₅₃ to Y+ relay. This signal causes the motor X to move to row C. When row C is reached, K₃ energizes causing Y+ to drop and K₅ to de-energize position. The next cam 8 signal now is directed to X+ relay. This entire sequence is repeated until the entire tray is canned. When the tray is in the 1F position, since K₄, and K₄A are energized, K₇ is energized through K₄₃. This causes the synchronous motor to stop (K₇₇) and the (Y-) relay to be energized, through K₇₃. The Y-relay causes the Y motor to run in the minus direction through its self-holding X₋ contact, K₁₇ and K₇₇. This motion is continuous until row A is reached at which time, due to relay K₁, (K₁₇) relay K₇ is de-energized and the cycle is completed.

In the manual mode, S₁ disconnects all appropriate lines of the automatic operation. Push-button switches SX+, SX₋, SY+ and SY₋ are operative and cause the tray to move in the appropriate direction when any one is energized. When the tray reaches any of the end positions the
corresponding switch, e.g. SX+ in the 10th position, is inoperative. Also, S1 switch is used to stop the automatic cycle at any given moment. By returning the S1 switch to the Auto-position and depressing the START push-button switch the cycle resumes.

When S1 switch is in the MANUAL position and S2 is switched to HOME position the tray returns to the origin (1A position).

The electro-optical system, the second electronic unit, is shown in Figure 6. The detailed drawing of the photomultiplier wiring in the detector channel is shown in Figure 7.

The stream containing the sample is illuminated from the mercury arc source through a BG 12 blue transmitting filter and a dark field condenser. The cell fluorescence is detected through a GG 14 yellow barrier filter by a 1P21 phototube. An operational amplifier with a pass band from 400 to 20000 hertz is used to amplify the electrical signals. This amplified signal is fed into the counter and also is monitored by the oscilloscope. The amplifier, oscilloscope, counter and phototube power supply are standard IRL laboratory instruments.

The counter counts the fluorescence signals observed with each sample and feeds the counts through an interface into the Instrumentation Research Laboratory LINC computer. At the beginning of the next injection the counter is reset and the new count begins. This cycle is repeated until the entire scan of the plate is completed. All the counts are stored in the computer and at the end of the scan the teletype prints out the tabulated counts.
It would be possible to perform data-analysis on the signal amplitudes of each sample by connecting a data analyzer at the output of the amplifier. Histograms could be derived and other more sophisticated data manipulations be performed by the computer depending on the characteristics of the various samples and the desires of the researcher. All this data could be stored in the computer memory for immediate or future reference. Little of this type of data processing has been performed on this contract due to lack of time and funds. However, it seems to be quite feasible and valuable information could result from amplitude analysis of samples under investigation.

In addition to the Figures mentioned above Figures 8, 9, and 10 are included with this report to provide sufficiently detailed working drawings so that a second unit could be constructed if desired. Figure 8 includes detail of the optical channel, and Figure 9 of the slider and syringe (moving arm) and plate holder, while Figure 10 gives a plan view of the entire unit.
Figure 9

Assembly of Moving Arm and Tissue Plate Holder
Operation

The following procedure is used for setting up and performing an experiment on this instrument.

1. Plug in the following components.
   1.1 Oscilloscope
   1.2 Counter
   1.3 Light Source Supply
   1.4 Computer Interface (Optional)
   1.5 Teletype (Optional)
   1.6 Tray Controller
   1.7 Phototube Power Supply
   1.8 Amplifier Power Supply

2. Turn on all instruments except the phototube power supply.
   Press button to start the mercury arc lamp.

3. Make system interconnections and connect the two saline solution reservoirs as shown in Figure 6. Turn on the vacuum. Focus and observe the stream through the microscope eye piece (45 degree mirror in appropriate position) and objective (filter removed). If no stream is visible after the system is in focus, the detector channel orifices are plugged and the optical channel assembly must be cleaned. If a stream is visible, move the optical table (Z adjustment) so that the glass capillary tube is just outside the field of view. Adjust the light source housing so that the back of the condenser is entirely flooded with light. Observing the stream through the eyepiece adjust the dark field condenser so that a dark, diffuse spot is located with its center
on the flowing stream. For this adjustment the Z and Y axis of the condenser stage must be used. When this is achieved move the condenser back and forth with the X-adjustment of the condenser stage while viewing through the eyepiece until the dark spot diminishes and a bright dot intercepts the liquid stream. When the spot appears to be the brightest possible the optical system is aligned properly. Insert the objective filter in position and refocus. Place a tray on the sample holder with a fluorescein solution (10^{-6} molar) and depress the start button on the tray controller (Auto-manual switch is in Auto position). Observe the stream. Immediately after each injection a yellow green line should appear in the field of view, becoming narrower with time and almost disappearing before the next injection. If the duration of this line is too long or too short adjust the elevation of the outer supply reservoir until the injected solution (fluorescein) nearly disappears before the next injection. Turn the phototube supply on (be sure the objective filter is in position) rotate the mirror on the microscope stage to the phototube position and observe the signal on the oscilloscope derived by the injection of the fluorescein into the channel. The counter at this point should show a large count (in the thousands). Readings should be reproducible within a few percent from one well to the next, and the signal on the oscilloscope should show a general increase in the signal level while the fluorescein is passing by, rather than large amplitude spikes (if this is not the case, adjust Z position of optical channel, and if necessary disassemble and clean the nozzle). Now return the tray in its home position and place the tray to be assayed in the tray holder. The instrument is now ready for operation.
Engineering Aspects and Evaluation

As with most new instruments, this equipment has required extensive trouble shooting and many modifications during the process of development. There has been a tendency for the small nozzles in the optical channels to clog. Sometimes this results in a flow stoppage which is easily detected and can be quickly remedied by the technician operating the unit. However at other times there is only a partial clog which results in erratic operation. In this case erroneous counts may be generated. Unfortunately there is no easy way to tell if such a partial clog has occurred.

It is necessary to cover the samples with an immiscible fluid of low vapor pressure to prevent evaporation of the suspending liquid. Some of this cover fluid clings to the outside of the syringe and is washed off into the optical channel. When heavy mineral oil was used as this fluid the instrument would clog quickly, presumably because high viscosity globules of oil became lodged in the nozzles. Use of a lighter mineral oil (#3 mineral oil: Van Waters & Rogers) minimized this problem, particularly when most of the oil was removed with a Pasteur pipette before analysis to minimize oil contamination. However use of any oil seemed to reduce the number of counts for a given sample slightly with respect to the count when no cover oil was used.

A wash cycle after each sample injection was included in the original designs. However during the initial debugging procedure various syringes were tried in an effort to get satisfactory operation,
and the most successful was not set up mechanically for the wash cycle. Later an attempt was made to change the syringe so as to reintroduce the cycle, but proper adjustment proved difficult and time consuming. Rather than delay the testing program we decided to continue without the wash since most of the required data could be obtained using alternate wells, filled with Hanks solution, as a wash.

It is sometimes difficult to set the signal threshold properly. (The threshold is the signal amplitude signals must exceed to be counted.) The cells give signals whose amplitude varies considerably. Superimposed on these signals is background noise generated by statistical variations in the number of quanta striking the phototube. This background noise is low when most of the fluorescein is in the cells. It is higher when the cells lyse and the fluorescein is in solution. As cells lyse the number of cells giving signals above the threshold level decreases, but the number of spurious signals from the background may increase if the threshold is set too low. In such a case it may be hard to discriminate between positive and negative responses.

It appeared that this condition could be improved if a more intense light source was used. Consequently tests were made comparing characteristics of the signals from a suspension of cells run through this unit, illuminated by its mercury arc, with those from the same suspension run through a similar optical system illuminated by an argon ion laser. The laser used (Coherent Radiation Model 54) is capable of increasing the effective illumination by between one and two orders of magnitude.
Results of these tests are shown in Figure 11. Curve A in this figure shows the pulse height distribution of signals from the laser illuminated system for a cell suspension incubated for 2 1/2 hours with a serum with which it was not expected to react. (By pulse height distribution we mean a plot of signal amplitude on the x axis versus a number of signals in a given narrow amplitude range on the y axis.) It is obvious from this plot that there is a wide distribution of signal amplitudes. Curve B shows the pulse height distribution for the same cells incubated with an incompatible serum for the same time. The large difference between the two curves indicates that threshold setting would not be critical. The threshold can be visualized as a vertical line adjustable to any point along the x axis. The relative number of counts is given by the area of the curve to the right of the line. No matter where the line is positioned the area under curve A is going to be many times the area under curve B. Results of similar tests of the present system using the mercury arc are shown in curves C and D where curve C represents negative reaction, and curve D positive reaction. Most of the "signals" near the origin are probably caused by noise spikes. It is obvious that here the position of the threshold could be very critical. It is probably desirable to move the threshold to the right for greatest differentiation between the two specimens but the total count falls rapidly when this is done and may be too low to be statistically meaningful. Thus the threshold adjustment must be made carefully and the proper setting may vary from sample to sample. This is undesirable in an instrument to be used in a clinical laboratory setting. In our
Figure 11
judgment future work with this unit might well include funds for replacing the mercury arc source with a reasonably powerful blue laser.

The electro-pneumatic drive system used in the ACTS requires rather delicate adjustments to function properly. In addition it is difficult to change the operating cycle and some desirable adjustments cannot be made at all. If we had been able to build a second system, as we originally proposed, we would probably have chosen to use an all-electric system with stepping motors rather than pneumatic actuators. Such systems are capable of almost infinite flexibility, and are easily adapted to computer control. Such flexibility would have increased the efficiency of our experimental design work markedly. In addition the system would be easily adaptable to such related tasks as dispensing fluids into the Terasaki trays, removing supernatant after incubation, and incorporation of wash cycles. It would also make it possible to vary the timing of the sample injection into the optical channel, and thus minimize disturbance of the sheath flow.

A set of runs were made in an effort to determine the length of time reliable operation could be maintained. Previous to these runs our subjective opinions were that clogging problems occurred every few plates. That may be true at times; however during the reliability tests the instrument operated quite reliably. Tests were run on six different days. One day we had trouble removing an initial clog but after it was removed the instrument ran through all six trays prepared without trouble. A third day there was a partial clog after five plates. The
three other days no clogs were experienced while running through all the trays available - six trays one day, eight another, and nine the third. An average of eight to ten trays between clogs should be possible after the unit was placed in routine operation, but care would have to be used to keep dirt out of the system. Operation in filtered air, using "clean room" techniques, would be desirable.

If we were initiating system development now we would seriously consider eliminating the present optical channel, observing the samples directly in the Terasaki plate wells. This would require a rather complicated mechanical scan if we had to observe individual cells. However it should be possible to provide the needed information by observing the total fluorescent output from the sample after withdrawing and discarding the supernatant. The cells tend to stick to the bottom of the well, and so should remain behind while the supernatant is removed. Thus high total fluorescence should correlate with high counts in the present system. In this case a speeded up version of the present mechanical movement of the Terasaki plate could be used resulting in faster system operation, and the clogging problems associated with the present pickup and delivery system would be eliminated.

Biological testing and evaluation

At the beginning of the test period we planned to conduct tests in thirteen biological areas. These areas, together with results when available, are discussed below. Many of them, of course, overlap the engineering problem areas discussed above. It should be noted that only selected results can be shown. A total of several hundred trays were
run through the machine in over five months of biological testing, interrupted only when shutdowns were imposed by instrumental problems.

1. Study the carryover of serum from well to well both in oil and without oil on the plate.

Table 1 shows typical performance with respect to carryover both with and without oil. Column 1 gives total counts from wells without an oil cover containing supposedly identical numbers of fluorescent cells. Column 2 shows counts from succeeding wells without cells. It can be seen that in this case on the order of 20% of the cells were carried over from one injection to the next. Similar results are shown in columns 3 and 4 showing performance under #3 mineral oil.

When a second well without cells was sampled the count dropped in about the same ratio. For example there were two blank wells after the fifth sample in each case above. The counts were 52 and 30 respectively.

Carryover shown in Table 1 is slightly higher than typical. A later analysis of several trays under oil indicated that, as long as cell counts were of the order of several hundred, mean carryover was between 10 and 15%. Relative carryover increased at lower count rates, but absolute carryover decreased. It was seldom over 20% for a properly functioning system when count rates on wells containing cells remained above 200, a properly functioning wash cycle would eliminate any carryover problem.
<table>
<thead>
<tr>
<th>Wells with Cells</th>
<th>Succeeding Wells w/o Cells</th>
<th>Wells with Cells</th>
<th>Succeeding Wells w/o Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>853</td>
<td>222</td>
<td>654</td>
<td>132</td>
</tr>
<tr>
<td>918</td>
<td>222</td>
<td>623</td>
<td>136</td>
</tr>
<tr>
<td>916</td>
<td>202</td>
<td>642</td>
<td>134</td>
</tr>
<tr>
<td>1026</td>
<td>246</td>
<td>648</td>
<td>164</td>
</tr>
<tr>
<td>925</td>
<td>232</td>
<td>712</td>
<td>162</td>
</tr>
<tr>
<td>856</td>
<td>142</td>
<td>617</td>
<td>114</td>
</tr>
<tr>
<td>937</td>
<td>211</td>
<td>642</td>
<td>125</td>
</tr>
<tr>
<td>1050</td>
<td>231</td>
<td>682</td>
<td>112</td>
</tr>
<tr>
<td>902</td>
<td>181</td>
<td>662</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 1

Cell Counts and Carryover With and Without Oil
2. Study degree of reproducibility.

Reproducibility, as illustrated in Table 1, was often very good so long as observations were made on a given sample with the same incubation times. Count rates on all samples, even controls using compatible sera, usually went down with time, as the fluorescein slowly leaked out of the cells. However on a given run variations in count rates of more than about ± 20% for samples with counts of over 100 per prima facie evidence of instrumental problems.

3 and 4. Study the effect of varying incubation time, serum volume, and serum strength on the results of the tests.

Table 2 shows average results for a typical series of tests over a period of time using two different amounts of serum. As shown in the table relative strong reactions are usually well underway within an hour. Other tests have shown readable difference after only 30 to 40 minutes incubation. The counts with respect to control on the positive sera decrease with time but are relatively constant between one and a half and two and a half hours.

As shown in Table 3 the relative strengths of sera may be different in the ACTS than with visual methods. The serum graded moderate to weak by visual observation appeared to be very strong in the instrument. Also this serum appeared to give better results at a lower concentration than at a higher one.
Table 2

Mean cell count (% of control) as a function of time of incubation, volume and strength of serum. Test conditions: 1 μl of cells and 4 μl complement, room temperature incubation.

<table>
<thead>
<tr>
<th>Serum Strength vs HLA5 Antigen (Manual method)</th>
<th>Serum Volume (μl)</th>
<th>Incubation Time (hrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Strong (1027D)</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Strong (1063C)</td>
<td>1</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>Moderate to weak (1079A)</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>Weak (1100C)</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>93</td>
</tr>
</tbody>
</table>
Table 3

Effect of Serum Volume

Test conditions: 1 μl cells, 5 μl complement, room temperature incubation for 2 hours.

<table>
<thead>
<tr>
<th>Antiserum Specificity</th>
<th>Volume μl</th>
<th>Counts Relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HLA1- 2+ Cells</td>
</tr>
<tr>
<td>HLA1+, 2-</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>81</td>
</tr>
<tr>
<td>HLA1-, 2+</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>
In the tests recorded in Table 2 one \( \mu l \) of serum usually took longer to react but gave slightly lower final counts than two \( \mu l s \). On the other hand many tests like those shown in Table 3 below indicate that counts relative to controls are lower with two \( \mu l \). Our general conclusion is that there is little real difference in final counts at 1 1/2 to 2 1/2 hours so long as the volume of serum is not so small as to prevent reaction, but reaction is slower at lower serum volumes.

5. Study variations in volume of complement with respect to amount and source.

This area was considered to be of low priority. There was some variation in amount of complement used, usually to keep total volume constant, but no specific tests were conducted on volume and kind of complement.

6. Study the variation in FDA concentration and time of FDA incubation.

This was important since, as discussed above (page 27), machine sensitivity was marginal and required maximum fluorescein content in the cells. We found it necessary to treat frozen cells with FDA after thawing, even if they had been treated before freezing. Our optimum procedure, arrived at after a number of tests, was to treat the cell suspension at a concentration of about 4000 cells per microliter with enough of a 1/2\% solution of FDA in acetone to make the final FDA concentration about 10 parts per million, incubate for fifteen minutes at room temperature in the dark, spin down, and resuspend the pellet in Hanks solution with 3\% BSA to approximately the original concentration.
This procedure gave good cell fluorescein content without leaving excessive fluorescein in the diluent.

7. Study the effect of elimination of BSA from the diluent, since it has been reported that it may inhibit FDA uptake.

Comparisons were run but no consistent inhibition was found. Since BSA does help protect the cell wall integrity, most of our testing was conducted with 3% BSA in the diluent.

8. Study the sensitivity of the method with respect to titers as compared with the manual method.

Only preliminary work was done in this area. No conclusive results were obtained. In order to study these phenomena fully an extensive testing program would be required. Unfortunately the instrument reliability is not high enough to make such an extensive program meaningful at present, particularly in view of the amount of time and money which would be needed to complete it.

9. Study the sensitivity of the method for the cross match in kidney transplants.

Our major hope in the preliminary stages of development was that the unit would be able to detect weaker interactions than was possible with standard techniques, and in fact preliminary tests on a non-automated system indicated this would be the case (3). However, tests made with weak sera on the ACTS unit showed no obvious improvement over visual methods. It would be desirable to repeat these tests with a laser light
source, giving a higher signal to noise ratio, to determine if under these conditions the expected improvement in detection would occur.

10. Compare the routine cytotoxicity with the automated one and with the inhibition method of Dr. Lucas. Comments under items 8 and 9 apply here.

11. Study the effect of Ficoll prepared cells as compared to the dextran sedimented cells. (Comments under item 8 apply here.)

12. Search for other oils since the routine mineral oil does not function well.

As noted on page 13, #3 mineral oil is much more suited to the requirements than the heavy mineral oil previously used. Another even lighter oil, essentially a purified kerosene, also gave acceptable results, but was more difficult to use, since the oil splashed out of the wells during the mixing cycle prior to sample pickup.

14. Compare manual phenotype versus phenotype obtained using the machine.

A start was made at machine phenotyping. However the problems listed above prevented its completion. Some differences might be expected between ACTS and routine methods, in view of the differences in apparent serum strengths noted.
GENERAL CONCLUSIONS

As a result of the tests to date it appears that:

1. The system can detect strong reactions quite readily in less than one hour. Weaker reactions may take about as long as the two to two and one-half hours usually used in the non-automated tests. When 1 μl of serum is used, weak reactions are probably not detected any more readily than with visual observations. Higher concentrations of serum (2 μl are used in most of the visual work) might improve this situation. Use of a strong light source (laser), giving a higher signal-to-noise ratio, would probably also be helpful.

2. The system will usually operate for several plates without trouble. However there is no assurance that it will do so and it is sometimes difficult to determine whether the readings are correct. Most problems have to do with clogging of the small nozzles in the flow system.

3. The system is limited to some extent by the fact that the threshold must be set near the noise level, so that sometimes noise pulses are mistaken for cells. As mentioned above, a more powerful laser light source would be helpful in this regard.
4. There are a number of delicate adjustments which require special skill. The combination of this with the clogging and threshold problems indicate that while the system is promising and could eventually provide a very worthwhile addition to the tissue typing field, especially if certain relatively minor modifications can be made, it is not yet ready for widespread clinical use.

5. If further development work is possible the following specific steps should be considered.
   a. Substitution of a laser light source for the mercury arc source, as mentioned on page 15.
   b. Conversions of the drive mechanisms from the present electro-pneumatic actuator to computer controlled stepping motors as mentioned on page 16.
   c. Elimination of the present delivery system, and observation of the total sample directly on the Terisaki plate, as mentioned on page 30.

Acknowledgments

The work done under this contract would have been impossible without previous and continuing associated work under National Aeronautics and Space Administration grant NGR-05-020-004 and NIH Grant 17367. We would also like to acknowledge the technical assistance and support of Ann Coukell and Gloria Dahm in the testing program.
REFERENCES


APPENDIX I

The achievements of the project and the extent to which it has met its desired goal are clearly and fully discussed in the main body of the report. I think the results demonstrate the feasibility of constructing a fully automated cytotoxicity typing system more or less along the lines we had originally envisaged, even though it did not prove possible to produce a fully operational system by the end of the contract period. As work on the system has progressed it has become clear that there are several modifications in the design as originally envisaged which would be desirable. Perhaps the major one, as indicated in the report, is to change from an electromechanical system with pneumatic actuators to an all electric system using computer controlled stepping motors. Other possibilities mentioned in the report include the use of a laser as the light source and the possibility of a mechanical scanning system for direct optical reading through the wells of the microtest plate without removal of the cells. I think it should be mentioned that a major reason for not having produced a fully operational system was the lack of funds, rather than the lack of time. I think that it is probable that, had we been funded for building a second prototype, this could have been done incorporating at least some of the suggested modifications, while at the same time allowing more intensive investigation of the properties of such a system as far as the biological testing was concerned. I believe that this principle of parallel development on the engineering and the biological side of such projects is a most important one, and
that although the initial sum of money to be spent may be more, the overall return on the investment may turn out to be substantially greater in the long run.

It is my feeling that many of the remaining problems of the system could be solved with the greater flexibility of a stepping motor controlled mechanical sampling device. Simple changes in the program, which could be done using computer software, could deal with the introduction of appropriate washing cycles, the removal of supernatant fluid before reading (which might substantially reduce the background and so avoid the need for a laser light source) and appropriate cleansing cycles to deal with the clogging problem. There is no doubt that the use of a laser would greatly increase the resolution of the system and that this would be extremely valuable for research purposes. The increased price, however, may not fully justify such a development if it were possible to achieve satisfactory results as indicated above. Any system which involves a direct reading through the wells of the Terasaki plates would, of course, be a great simplification. The difficulties in this case are connected with the problems of the background fluorescence in the fluid in which the cells are suspended and the problems of overlapping cells if an accurate cell count is desired from a scanning approach. This latter factor may not be important if one simply wants a quantitative measurement which is well correlated with the number of fluorescing cells. The former problem may be alleviated by removing the supernatant fluid before reading, in such a way as to leave the majority of the cells in the well. In this case it may even not be necessary to
have the mechanical scanning device. It may then be possible simply to take a direct reading through the well.

I think it is worth emphasizing that the approach to sampling from a Terasaki plate which we have used may have many applications quite apart from that to tissue typing. I believe that the use of the micro-test plates in such a system could be viewed as part of a programable automatic fraction collector and dispenser that disposes of the rather clumsy devices presently used with tubes for fraction collection. The output of material collected from the wells may, for example, be analysed for cell counts or for radioactivity in a variety of ways.

Walter F. Bodmer
Present Address:
Genetics Laboratory
Department of Biochemistry
University of Oxford
South Parks Road
Oxford, England