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Application of Cell Analysis and Sorting Techniques to Disease Detection

October 1, 1977—September 30, 1978

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J. C. Forslund*
E. T. Brake
L. S. Cram

*LASL Consultant.

APPLICATION OF CELL ANALYSIS AND SORTING TECHNIQUES TO DISEASE DETECTION OCTOBER 1, 1977—SEPTEMBER 30, 1978

by

J. C. Forslund, E. T. Brake, and L. S. Cram

ABSTRACT

Significant progress has been made on the development and characterization of a cell-mediated assay for bovine tuberculosis. Using a whole blood culture technique, lymphocyte proliferation in response to a purified protein derivative from *Mycobacterium avium* (PPD-A) or *Mycobacterium bovis* (PPD-B) was measured. The analysis was done by flow microfluorometry of fluorescently stained lymphocyte DNA. Samples from experimentally infected animals and field cases were evaluated using this procedure. Optimization of the protocol is reported and investigations into new diagnostic techniques are reported.

I. BACKGROUND

The objective of the cell analysis project at Los Alamos Scientific Laboratory (LASL) is to develop new and better biophysical and biochemical markers that are compatible with flow systems and to characterize these markers for detection and diagnosis of animal disease. As this technology is developed, it will be transferred to the appropriate users in the United States Department of Agriculture (USDA). The work at LASL is coordinated with similar work at the National Veterinary Services Laboratory (NVSL) at Ames, Iowa under the direction of Charles Thoen and work at the University of Minnesota under the direction of Donald Johnson. The Animal Plant Health Inspection Service (APHIS) coordinator for the cell analysis project is Kenneth Hook at NVSL.

II. INTRODUCTION

LASL scientists have developed unique flow microfluorometry (FMF) instrumentation that is suitable for monitoring a wide variety of cellular properties. Some of these properties are 1) cellular DNA content, 2) light scattered by cells from an exciting laser beam, and 3) cell volume. Since these measurements can be done on single cells at a high rate (up to 10^5 cells per min), it is possible to obtain a good statistical sampling even if only a few cells are different from most of the population. This instrumentation is being used to obtain a better understanding of the immunological process that, in turn, can be applied to disease monitoring.

Measurement of lymphocyte proliferation in response to stimulation by a specific antigen is an accepted method of disease monitoring[1], and this

method has been adapted to the FMF instrumentation. Lymphocyte stimulation is monitored by staining control and stimulated sample with a fluorescent DNA stain and looking for increased DNA content in the antigen-stimulated sample as compared to the control sample. This method of sample analysis has several advantages over triated thymidine analysis.[2-4]

Previous reports[5-9] have described details of the cell analysis and sorting program as it applies to bovine tuberculosis. These reports contain program justification and supporting data. A recently published paper[10] summarizes the conclusions on our Newcastle disease virus (NDV) work. An invited paper has been submitted for publication describing some of the bovine tuberculosis results.[11]

Since January 1978, L. Scott Cram has been at the Max-Planck-Institut für biophysikalische Chemie in Göttingen, West Germany. This particular lab was chosen because of its unique application of spectroscopic techniques to flow systems and its international reputation in this field. While there, Scott Cram helped organize and participated in the Sixth Engineering Foundation Conference on Automated Cytology held in West Germany.

III. BOVINE TUBERCULOSIS

A. Procedure Optimization

We believe that significant improvements are possible in detecting cell stimulation. Searches for these improvements will follow two general paths: 1) keeping up with the immunological literature to gain a better understanding of immunological processes, and 2) testing various aspects of the procedures being used to optimize them. The first path could lead to major changes in the test while the second path is more likely to result in smaller changes.

It has been shown[12] that under hyperthermic conditions the mitogenic response of human lymphocytes is earlier and elevated. The whole blood culture technique utilizes a 37° incubation temperature[13], which is 2° lower than bovine body temperature. Therefore, two higher incubation temperatures, 39°C and 40°C, were investigated using blood collected from animals at the NVSL. Animals with a known positive response to PPD-B (#8067 and #3), were used in several experiments over an 11-week period. Within this time period each animal showed variation in the maximum amount of response to PPD-B, as well as the shape of the stimulation-difference-vs-time curve. The results for 37°C and 39°C incubation temperature are plotted in Fig. 1. Note that for the same culture conditions differences in peak response time and curve shape occurred between the two animals. Thus, the optimum incubation time was different for each animal under identical culture conditions. The two animals were similar in that both achieved a given magnitude of response approximately 24 h earlier at 39°C than at 37°C. However, a greater maximum response was eventually achieved at 37°C for both animals. When these two animals were compared at 37°C and 40°C incubation they again differed, but in neither case was 40°C incubation better than 37°C incubation. Cultures from animals with a negative PPD-B response at 37°C also showed no response at 39° or 40°C incubation. The magnitude of the PPD-A response at 39°C for animals #8067 and #3 was consistent with 37°C data, whereas the peak PPD-A response occurred 11-16 h later than the peak PPD-B response for both animals. Due to the animal difference encountered in these experiments, no final decision regarding 39°C incubation has been made. If these animals are typical in their response, it appears that use of 39° incubation would shorten the incubation time 24 h over the presently used 135 h at 37°C. However, if time is less important than maximum response, 37° remains the best incubation temperature.

B. Data Handling

Bovine tuberculosis-lymphocyte stimulation data from the last three years has been removed from bulky card storage to computer disk and magnetic tape. This library of data is easily referenced and is available for further analysis should new methods of computer analysis be developed. In addition a new method of data acquisition has been developed for FMF. Data is collected directly onto computer disk instead of going through intermediary steps of paper punch and computer cards. This new system allows more samples to be run per hour on FMF and

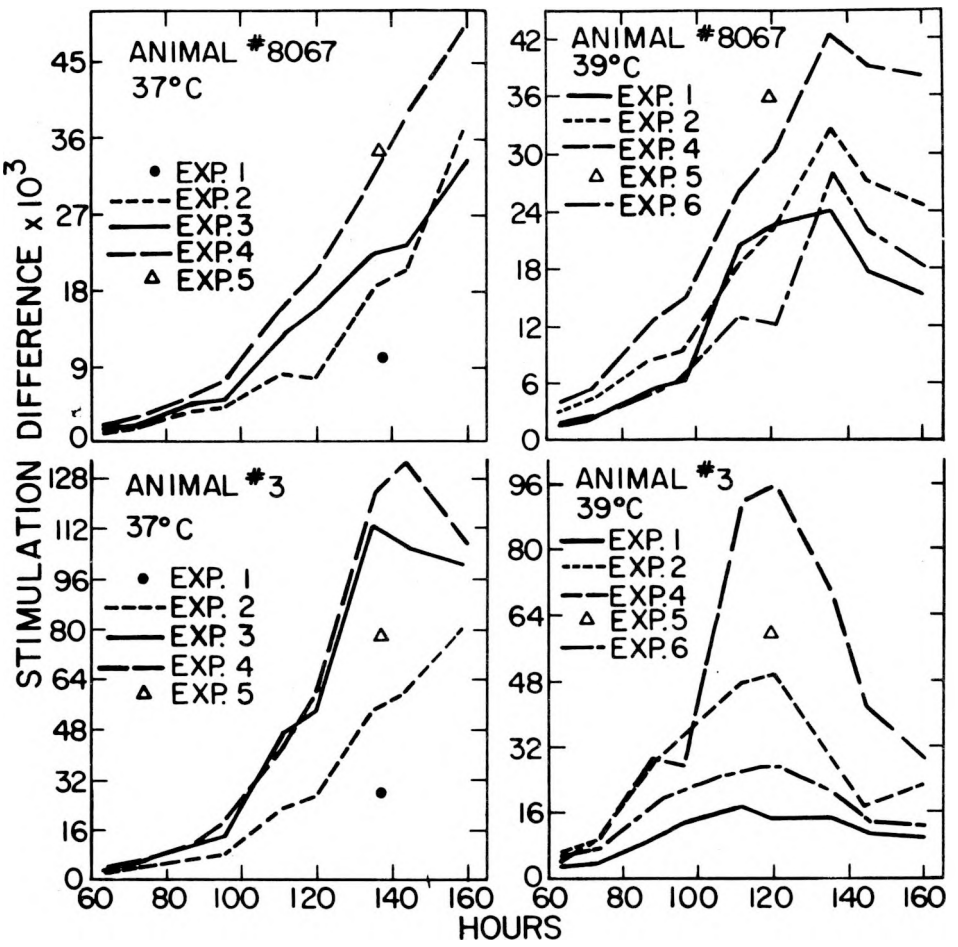

Fig. 1.

Comparison of cell stimulation difference vs time. Except for the noted change in incubation temperature, culture conditions were identical for all experiments. The six experiments were based on six different bleedings spaced one or two weeks apart over an 11-week period in time. (See discussion of data handling for definition of stimulation difference).

greatly shortens the time involved in analyzing the data to quantitate lymphocyte stimulation.

We have quantitated lymphocyte stimulation in two different ways. Both ways require normalization of raw data[13] before calculations are made. Currently, we are using a stimulation ratio that is calculated by dividing the number of stimulated cells in a PPD-B culture by the number of stimulated cells in a control (no PPD-B added) culture. However, the data in Fig. 2 and Table I show that small changes in the number of stimulated cells in the control can have a large effect on the stimulation ratio calculated. In contrast, calculation of a stimulation difference is not so drastically affected by these changes in the control-stimulated counts. The background of non-specifically stimulated cells in the control culture is subtracted from the stimulated cell count in the PPD-treated culture to yield the number of cells specifically stimulated by the PPD. Stimulation ratios continue to be calculated for comparison to other data. However, the stimulation difference seems to be a more meaningful indicator of numbers of specifically stimulated lymphocytes and is also being routinely calculated.

C. Experimental Infections

A cooperative experiment for inducing *M. bovis* infection by exposure to an infected animal has continued. Blood samples are concurrently examined at

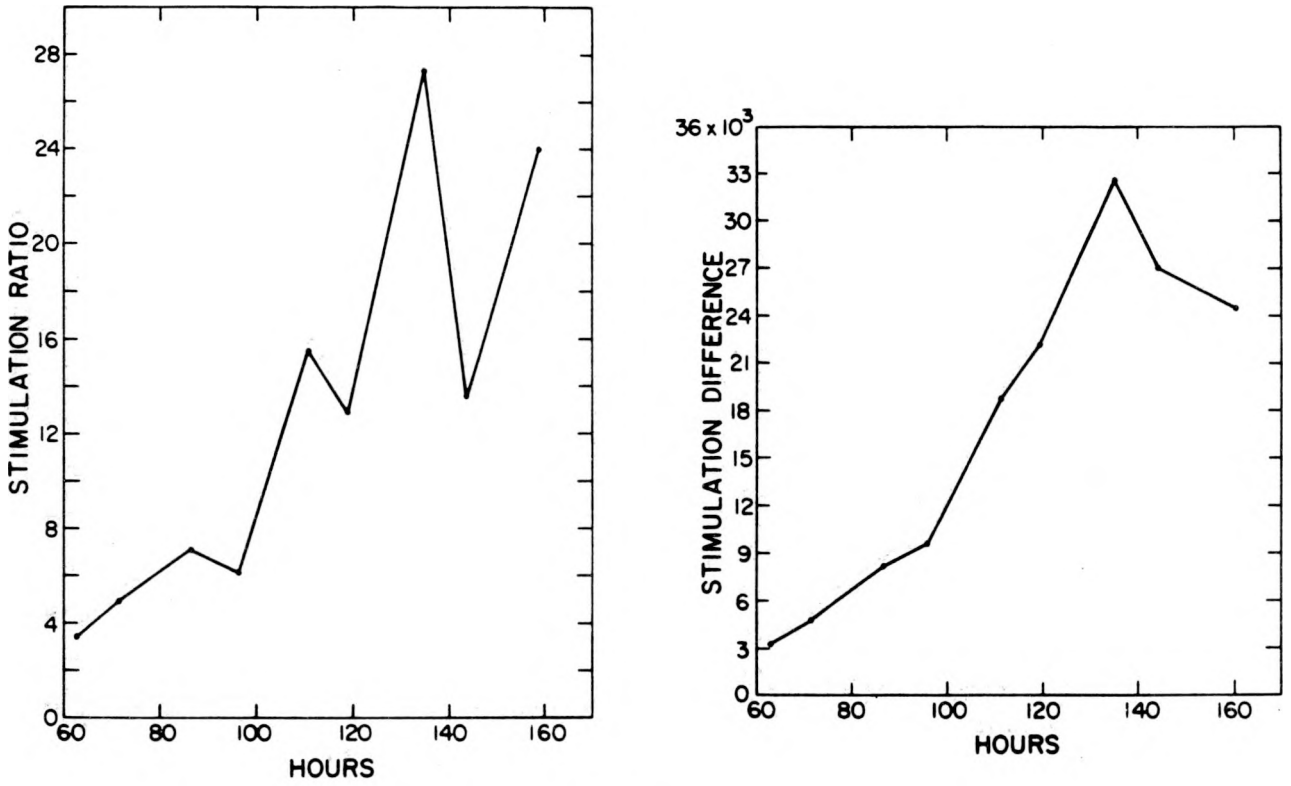

Fig. 2.
Comparison of stimulation ratio and stimulation difference. The same raw data from animal #8067, experiment #4 (39°) was used to calculate both the ratio and difference.

TABLE I

	Hours After Antigen Addition								
	62.75	71.5	86.5	95.5	110.75	119.25	134.75	143.5	158.75
Control stimulated counts	1324	1181	1314	1817	1297	1860	1239	2138	1070
PPD-B-stimulated counts	4592	5892	9372	11310	20041	24071	33829	29174	25633
Difference	3268	4711	8058	9493	18744	22211	32590	27036	24563
Ratio	3.47	4.99	7.13	6.22	15.5	12.9	27.3		

NVSL, LASL, and the University of Minnesota. Additional data on that experiment is reported here. Animal #8067, which had become positive as previously described,[9] and #97, which had remained negative, were kept in the experiment, whereas animals #95 and #17 were slaughtered for a TB school at Veterinary Services Laboratory, Ames, Iowa. These latter calves were replaced by animals #36 and #41, whose first bleedings were received at LASL on December 29, 1977 (50 weeks from the start of the original experiment on January 12, 1977). As can be seen in Fig. 3, animal #8067 remained positive from 32 weeks until the last data point at 72 weeks. However, large fluctuations in PPD-B stimulation ratios were observed as had been the case with animal #17 (Ref. 9). Animal #97 remained essentially negative (stimulation ratio < 3.0) except for 2 bleedings at 60 and 62 weeks. This short positive response is very similar to the one observed at 10 and 12 weeks in this animal. The

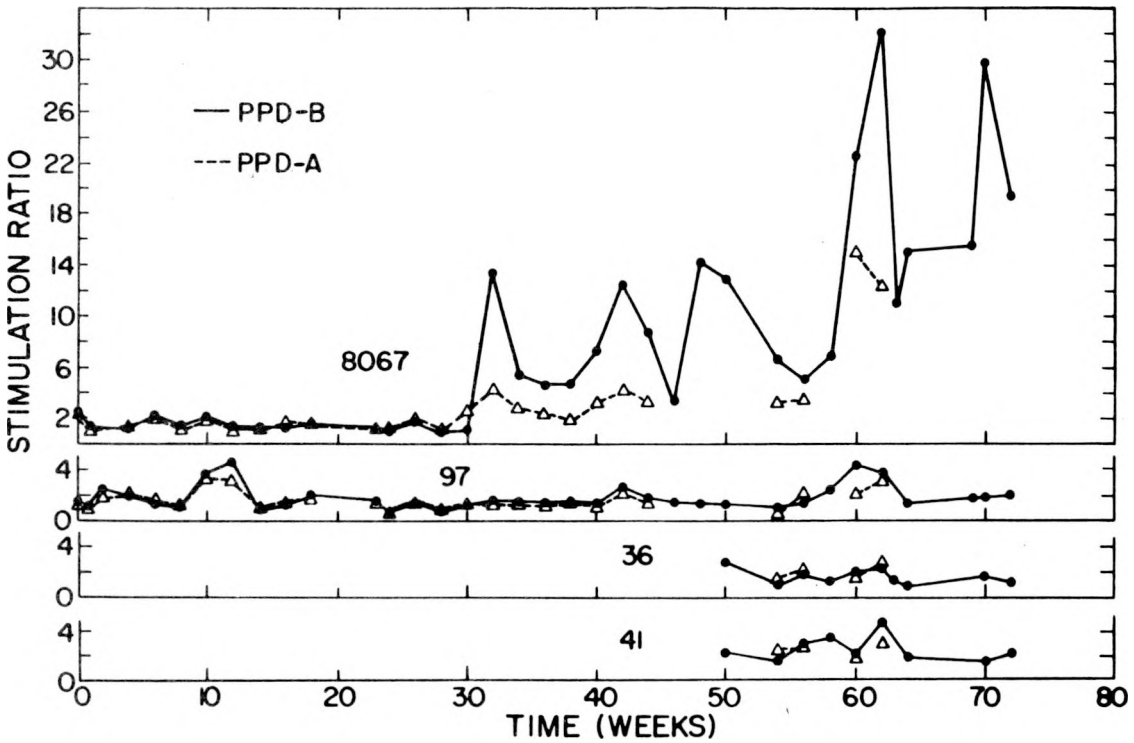

Fig. 3.

Stimulation ratio history starting January 12, 1977, of four laboratory animals housed together. Animal #8067 and 97 were housed with a TB-positive animal from 0 to 38 weeks. All animals were TB negative at start of experiment and #8067 became positive as previously described.[9] Blood was incubated at 37°.

significance of these two brief periods of higher response is not known. Animal #36 remained negative and animal #41 essentially negative (one positive response at 62 weeks) from 50 to 72 weeks (see Fig. 3).

Beginning August 11, 1977, biweekly bleedings were also received at LASL from five additional animals housed at NVSL, Ames, Iowa. These animals had been purchased from a TB-infected herd and brought to the laboratory. They were kept separate from # #97, #36, and#41. As can be seen in Fig. 4, animal #3 maintained a positive response to PPD-B over a 42-week period. Again, fluctuations in response like those seen in animals #17 and #8067 were observed. Animal #3 had an unusually low PPD-A response for a PPD-B positive animal. The PPD-B response of animal #9 was essentially negative except for weak positive response at 8-12 weeks and an isolated positive response at 32 weeks. Animal #9679 remained negative except for a single positive response at 12 weeks. Animal #9681 remained negative with a similar single positive response at 32 weeks. Animal #9682 was consistently negative (see Fig. 4).

The causes of variation in the stimulation response of a single animal from bleeding to bleeding remain unknown and need to be understood or eliminated if the test is to be made more reliable. Replicate samples from a single bleeding give essentially the same results if fluctuations in the control are taken into account (see Data Handling section). Therefore, we do not think the variability is caused by the instrumentation. It is well known that immune system functions can be significantly altered by challenges to the immune system. So it is possible that the variations from bleeding to bleeding that have been observed in the stimulation response in positive animals can be attributed to additional immunological challenges such as a TB skin test or an unrelated disease. If this

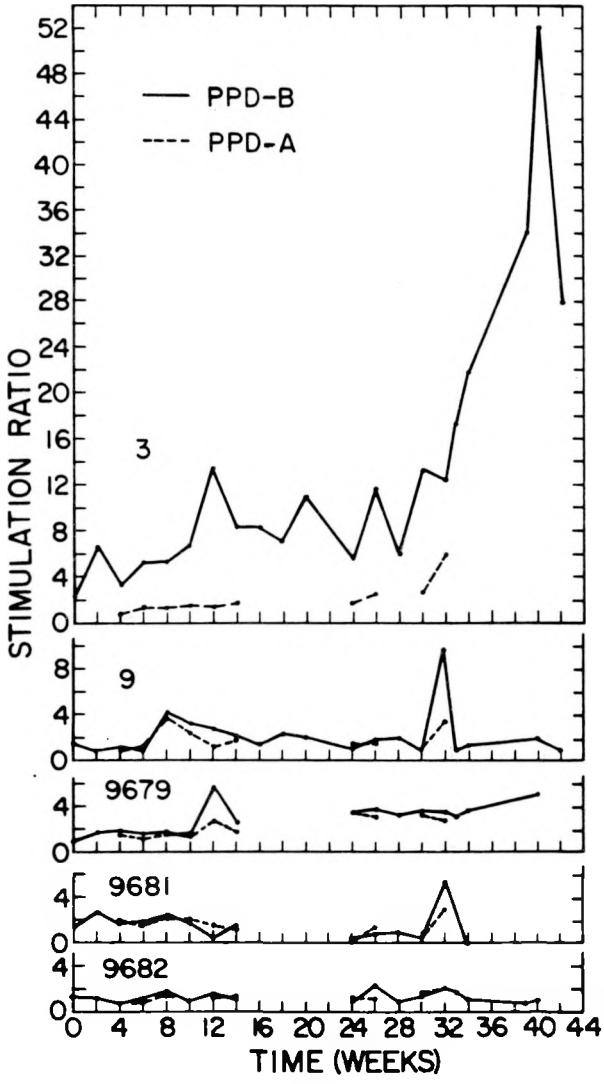

Fig. 4.
Stimulation ratio history starting August 11, 1977, of five animals purchased from a TB suspect herd (Ref. 9-103) and housed together at NVSL, Ames, Iowa. These animals were housed separately from those in Fig. 3. Blood was incubated at 37°.

is true, then it would be desirable to obtain some measure of the general state of the immune system. If indicators of this sort could be found, it would be possible to minimize the fluctuations in responses and make the test more consistently accurate.

The biweekly data collected on these two sets of animals (a total of nine animals) provides valuable information concerning variability in lymphocyte stimulation within a single animal over several months' time. Some indication of the ease of spread of TB from one animal to another has also been obtained. Last, the data base on positive animals #8067 and #3 allows for better evaluation of new approaches to diagnosis using blood from these animals in experiments

D. Field Studies

Personnel from LASL, University of Minnesota, and USDA-NVSL agreed that single bleedings of herds (considered to be in the high risk category of being infected with *M. bovis*) was not yielding data of much value. Therefore, it was decided to seek a small herd at high risk of containing tuberculous animals on the basis of previous testing. Such a herd was found, purchased, and isolated. It contained 24 adults and 32 calves. The calves were bled once and none gave a positive response to PPD-B stimulation. The 24 adults were bled seven times starting January 10, 1978, and ending March 1, 1978. Samples were sent to LASL, University of Minnesota and NVSL, Ames, Iowa. Tubes of blood were numbered 1-24 and the corresponding identity of each animal for each bleeding was not known until the 7 bleedings were finished. Many samples from the third, fifth and sixth weeks yielded poor data when analyzed by FMF (see Table II) even though the blood visibly looked good upon arrival. Blood from the seventh week was discolored and lacked viscosity and no usable data was obtained from this last bleeding. After all bleedings were complete, it was learned that the University of Minnesota personnel also had experienced many poor samples. When tube numbers of poor samples were compared, a high degree of correlation was seen between LASL and University of Minnesota data (see Table II). Such correlation suggested something was wrong with the blood prior to laboratory handling and analysis. The bleedings were made under severe cold weather conditions and it was hypothesized that either the blood got too cold or too hot (in effort to keep it warm in the car). Stimulation ratios obtained at LASL for the seven bleedings of the 24 animals are shown in Fig. 5. Note that data from poor samples are identified and should carry a low degree of confidence. Most of the animals were consistently negative by LASL analysis. Animal #42 CFC 6293 had an initial weak "positive " ratio but

TABLE II

CORRELATIONS OF POOR SAMPLES

DATE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1/3/78*																								
1/10/78			LOW CELL COUNT BUT PEAKS OK (LASL)																					
1/17/78			M											M										M
2/1/78	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
	B	B	B	B	B	B	B	B	B		B	B	B	B	B		B	B	B	B				
2/7/78																								
2/15/78	M	M	M	M	M	M									M	M	M	M	M					
	B	B	B	B	B										B	B	B	B	B					
2/22/78	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M								
	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B					
3/1/78	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
	NO DATA DUE TO POOR CONDITION OF BLOOD (LASL)																							

*32 samples from calves — one bleeding only

M = NO DATA REPORTED FROM MINNESOTA

B = POOR DATA FROM LASL SAMPLES

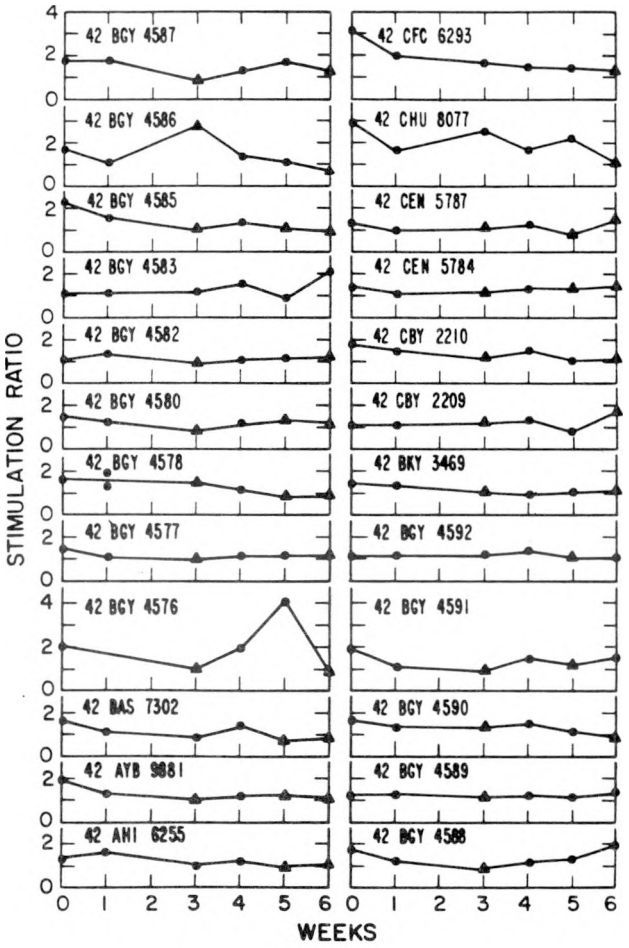

Fig. 5.
Stimulation ratio history (six bleedings) of 24 animals of a field herd (Ref. 10-2). Blood from the seventh week was not suitable for culturing so no data were obtained.

was consistently negative in subsequent bleedings. Animal #42 CHU 8077 gave somewhat elevated response but looks basically negative. The only animal that might be called positive by LASL analysis is #42 BGY 4576 and such a conclusion is weakened by the previously noted problem of poor samples. It was disappointing due to the time and effort involved that this herd yielded no definitive positive animals..

Samples from two other field herds were also analyzed. Only one bleeding was made in both cases. The stimulation ratios are plotted in descending order in Fig. 6. None from the herd of 20 animals was positive and one animal from the herd of 25 (no data from 2 animals) was weakly positive.

IV. OTHER APPROACHES

Changes in membrane fluidity are believed to be associated with lymphocyte stimulation. Since other membrane changes, such as nutrient transport, occur very soon after stimulation, it is likely that the membrane fluidity change is also rapid. Membrane fluidity can be quantitated by measuring the degree of fluorescence depolarization from a fluorescent molecule (probe) embedded in the lipid layer of the membrane. If the probe is moving freely in the membrane lipid (i.e., the membrane is very fluid), the polarized light absorbed by the probe will be depolarized before re-emission. On the other hand, if the probe is held rigidly in the

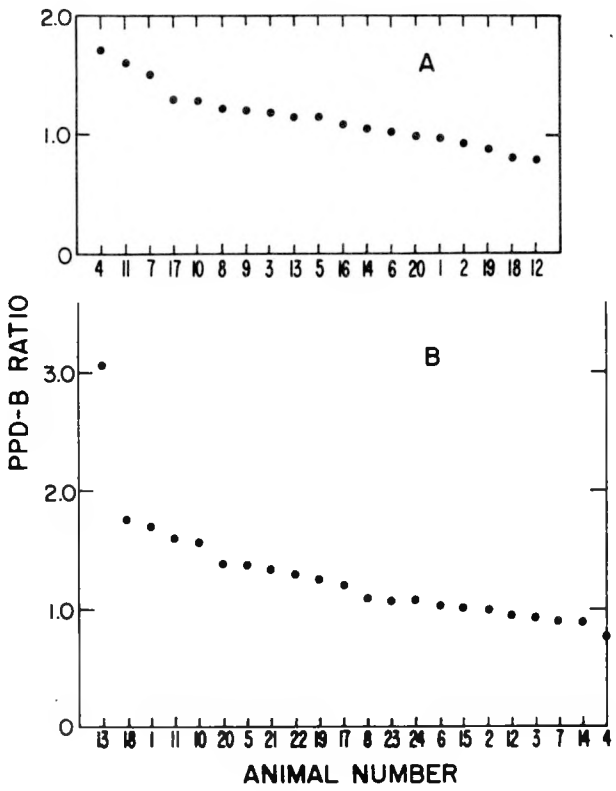

Fig. 6.
Stimulation ratio history of two field herds (Ref. (A) 9-279 (B) 11-1)plotted in decreasing order of stimulation ratio.

membrane, the polarized light will be absorbed and re-emitted before movement of the probe occurs. Therefore, the laser light will remain highly polarized.

Our goal has been to measure the membrane fluidity of individual cells while simultaneously measuring DNA content, which is known to increase after lymphocyte stimulation. The availability of some unique membrane probes, which do not move out of the membrane into the cytoplasm, have made this work possible. A dansylated glycolipid and a dansylated phospholipid were tested under different conditions for their staining suitability on several cell types. Both lipid probes were found to bind to the membrane and to remain in the membrane bilayer for a sufficiently long period of time for flow cell analysis to be made. Cellular DNA content is detected by staining with a new dye (a Hoechst derivative). We are currently optimizing the double staining procedure to determine which of the Hoechst dyes is most suited for the experiment as they all have slightly different spectral properties and behave different histochemically.

V. FUTURE ACTIVITIES

A. Improvements in Procedure

The use of whole blood cultures with our technique for FMF detection of PPD-B-induced lymphocyte stimulation has eliminated the time-consuming step of lymphocyte isolation from each blood sample. However, unlike propidium iodide-stained lymphocyte samples, stained whole blood samples often deteriorate upon storage at 4°C. This deterioration is evidence by broadening of the G_1 peak and a considerable increase in the amount of sample debris. We reported previously that addition of 5% ethanol to stained whole blood cultures gave fairly satisfactory preservation.[9] However, the effectiveness of ethanol preservation varies from animal to animal. Experiments will be conducted in the coming year to determine a more reliable method of preservation for stained whole blood samples.

Since bovine blood is not available continuously, especially from TB-positive animals, it is important to be able to preserve available blood for future studies. We plan to develop a suitable freezing method for whole blood so that long-term storage will be possible.

An additional part of the existing procedure, which we want to optimize further, is data analysis and storage. Areas of possible improvement include 1) modification of the present computer method of data analysis to a less time-consuming operation, 2) re-evaluation of possible methods for reporting the degree of lymphocyte stimulation, and 3) standardization of the reporting procedure with other workers in the field.

B. Earlier Detection of Cell Stimulation

Several other approaches are being considered which may shorten the time required to detect lymphocyte stimulation. These include measurement of changes in 1) cell staining properties, 2) esterase activity, 3) cytoplasmic viscosity, and 4)

membrane permeability, which have all been reported to occur a short time after antigenic stimulation. We plan to measure these parameters at various times after antigen (PPD-B) addition and to evaluate which measurement affords the easiest, earliest, and most sensitive measure of antigen stimulation of lymphocytes.

A potentially very exciting improvement in our analytical technique appears to be available based on recent investigations by Lalande and Miller of the Ontario Cancer Institute.[14] This development offers three attractions for our specific purpose: 1) a reduction in incubation time (12 h vs 135 h), 2) simplicity of cell staining, and 3) a staining procedure that is nontoxic. The bis-benzimadazole dye Hoechst 33342, when used at low concentrations, was found to stain responding cells brighter than nonresponding cells.[15] Optimum dye concentration seems to be about 2 μM. At these dye concentrations, the cell fluorescence is not a measure of DNA content as is true at slightly higher concentrations (10 μM). The procedure has been tested for both allogenic and mitogenic stimulation.

The first two advantages listed above are obvious and can be fairly easily adapted to our procedure. However, the reason for the difference in fluorescence intensity is not well understood and needs to be investigated. One idea that has been invoked to explain the fluorescence differences between responding and nonresponding cells is cell permeability. This does not satisfactorily explain all of the observations. Therefore, it will be important to investigate the reason(s) for the differences in cell fluorescence since other Hoechst derivatives are available that have potentially even better properties. To select which derivatives to test, it is necessary to have a better understanding of the cellular differences that are to be exploited.

The third advantage, nontoxic staining, offers even more exciting potential. To date we have been measuring the amount of DNA per cell as a marker for immune function without regard to the functional identity of the cells. It would be desirable to identify the functional subclasses of cells that are responding and to determine if the responses of some subclasses are more specific than are others. The effect of such a determination would be to greatly increase our signal-to-noise ratio or, in other terms, allow us to differentiate between a specific response to PPD-B by one functional subclass and any nonspecific response due to another subclass of cells. The nontoxic properties of Hoechst 33342 will permit us to sort early responding cells that should correspond to functional subclasses of cells responding to specific antigenic stimulation. This is believed to be a low percentage of the total number of cells that are in blastogenesis at 135 h. Once this early responding subclass of lymphocytes is isolated and characterized, it should be possible to develop other flow cytometry markers (light scatter, cell size, chromatin patterns) to aid in its identification.

It must be realized that, at present, the cell pool we are using remains poorly characterized as to the number, function, and specificity restrictions of its different cell populations. Helper T cells, macrophages, and suppressor T cells have, however, been implicated as necessary for responding cells to start DNA synthesis. Once these cellular relationships are known, several greatly improved diagnostic techniques should result.

Preliminary results from our laboratory show changes in esterase activity following lymphocyte stimulation. The method used for esterase detection involves the incubation of isolated lymphocytes with the nonfluorescent compound fluorescein diacetate (FDA). Viable cells take up FDA very rapidly. The enzyme(s) esterase then cleaves FDA into two acetate molecules and fluorescein, which is fluorescent and can be detected using the FMF.

Using the technique mentioned above for introduction of fluorescein into the cytoplasm of lymphocytes, Cercek and his coworkers have observed that the emission polarization spectrum of cytoplasmic fluorescein at a specific excitation wavelength (470 nm) changes after stimulation of human lymphocytes with the mitogen phytohemaglutinin.[16] This is probably due to a change in the viscosity of the cytoplasm after stimulation. We plan to investigate this parameter as a means of detecting lymphocyte stimulation.

Another possible means of rapid detection of lymphocyte stimulation is measurement of increases in the rate of nutrient transport across the cell membrane, which occur soon after lymphocyte stimulation.[17] We plan to measure amino acid transport initially, since this is probably the easiest

of the transport systems to measure. Measurement of glucose transport and potassium transport are additional possibilities.

One of the earliest events to occur in the process of lymphocyte stimulation is binding of the antigen to receptors on the cell surface of responsive cells. We plan to conjugate PPD-B to fluorescein and measure binding of the fluorescent conjugate to lymphocytes from TB-positive animals. If this measurement is adequately sensitive, very rapid detection of TB-positive animals should be possible.

It is necessary to further test the present diagnostic procedure (and other methods as they are developed) in carefully controlled field studies. Knowledge in the field of immunology is changing rapidly, and these discoveries should be applied in a timely manner so APHIS disease detection methodology will be the best available. Comparison of accepted methods with new methods in laboratory and field studies is an important part of this effort.

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