LA-8227-PR

Progress Report

Animal Disease Detection Using a New Rapid Method for Monitoring Antigen-Induced Lymphocyte Stimulation

October 1, 1978—September 30, 1979

University of California

LOS ALAMOS SCIENTIFIC LABORATORY

Post Office Box 1663 Los Alamos, New Mexico 87545

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This work was supported by the US Department of Agriculture.

LA-8227-PR
Progress Report
UC-48
Issued: June 1980

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ANIMAL DISEASE DETECTION USING A NEW RAPID METHOD FOR MONITORING ANTIGEN-INDUCED LYMPHOCYTE STIMULATION

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by

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ABSTRACT

Lymphocyte proliferation in response to bovine tubercular antigen was measured on blood samples from a herd naturally infected with TB. Determination of single-cell DNA content as a measure of cell proliferation was done by flow cytometry after staining with the DNA-specific stain, propidium iodide. Results of the field testing showed good agreement with other methods for TB detection. Significant progress on the development of a new method for TB detection was made. An increase in the rate of amino acid uptake can be measured within 14 h after treatments of lymphocytes from TB-positive animals with TB antigen. The enhancement does not occur after similar treatment of lymphocytes from TB-negative animals. Ways for optimizing the new test and investigations into other 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 for the 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 Donald Luchsinger 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

Our present method for the detection of bovine tuberculosis utilizes the unique flow cytometry instrumentation developed at LASL. Previous reports have described the details of this technique.[1-6] The method measures lymphocyte proliferation in response to a purified protein derivative (PPD-B) from Mycobacterium bovis. Proliferation is monitored by fluorescently staining lymphocyte DNA and by using the flow cytometer to measure the DNA content of single nuclei. Fluorescence intensity per nucleus is proportional to DNA content. PPD-B-treated cultures from TB-positive animals contain a larger number of cells that have more than one complement of DNA (i.e., proliferating cells) than do nonantigen-treated cultures from the same animal.

This report describes (1) improvements made in the detection technique described above, (2) a successful field study using this technique, (3) testing of PPD-B preparations from several different sources, (4) a preliminary report of a new technique for detection of antigen-induced lymphocyte stimulation that requires a culture time of only 14 h instead of the 6-day culture time necessary for the present method, and (5) a description of the future activities related to this project to be carried out at LASL in the next year.

III. ACTIVITIES OCTOBER 1, 1978 - SEPTEMBER 30, 1979

A. Improvements in the Flow Cytometric Technique

Our technique of using whole blood cultures for the flow cytometric 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 isolated lymphocytes, stained whole blood samples often deteriorate upon storage at 4°C. This deterioration is evidenced 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 improved sample stability[5] but was not effective for storage of samples from all animals tested.

We have now developed a very reliable method for sample preservation. To facilitate lysis of the red blood cells in whole blood cultures and entry of stain into lymphocyte nuclei, the propidium iodide staining procedure is normally carried out at 4°C in a low ionic strength solution. We have found that if the low ionic strength staining procedure described above is followed by addition of sodium chloride to a final concentration of 0.16 M (the concentration present in the culture medium) and ethanol to 5%, the stained whole blood samples are stable for long periods of time. Figure 1 shows that at room temperature, stained cultures stored in low ionic strength solution begin to degrade within 3 days and are completely degraded in 11 days. Duplicate samples stored with 0.16 M NaCl and 5% ethanol are very stable over the same time period. For prolonged storage, we recommend that samples be held at 4°C, which further guarantees their stability. Addition of sodium chloride alone with no ethanol is not effective in preventing the degradation.

Bovine blood, especially from TB-positive animals, has not been available on a continuous basis. To standardize testing procedures, it is important to have a supply of blood from known positive and negative animals. We have developed a suitable whole-blood freezing method that preserves lymphocytes and facilitates long-term storage. Blood samples are diluted with culture medium (RPMI 1640), and 5 to 15% dimethylsulfoxide (DMSO), a cryoprotective agent, is added. Two-ml aliquots are then frozen in plastic vials at a rate of 2.5 to 5°C per min. until a temperature below -80°C is reached. Finally the samples are immersed in liquid nitrogen for long-term storage.

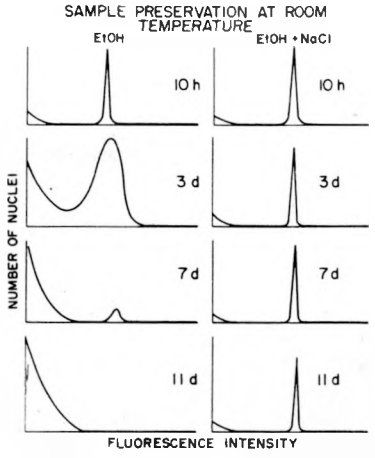

Fig. 1. Stabilization of propidium iodide-stained whole blood cultures with ethanol and NaCl. A whole blood culture was stained with propidium iodide (50 μg/ml) in 4mM sodium citrate at 4°C. To half of the stained culture, ethanol was added to a final concentration of 5% (1.1M). To the remainder of the culture, NaCl was added to 0.16M and ethanol to 5%. The samples were then held at room temperature and analyzed by flow cytometry at the indicated times.

Thawing of samples is rapid, 37°C with agitation until the last ice crystal has disappeared. The samples are then diluted with medium, centrifuged, washed, and resuspended in culture medium. Red blood cell integrity is not maintained using this procedure because the optimum freezing rate for red blood cells is much faster than 2.5 to 5°C per min.[7] Thus, the procedure also enriches for lymphocytes.

Variations in the (1) freezing rate between 2.5 and 5°C per min, (2) DMSO concentration between 5 and 15%, (3) cell concentration between blood dilutions of 1:2 and 1:10 with medium, and (4) serum concentration between 5 and 40% do not affect lymphocyte recovery (the number of intact cells present immediately after thawing) or lymphocyte viability. Viabilities are determined by response of lymphocytes in culture to stimulation by PPD-B for TB-positive animals, and phytohemagglutanin (a well characterized lymphocyte growth inducer) for TB-negative animals. Viabilities of frozen-thawed samples are compared to duplicate nonfrozen controls. Normal recovery of lymphocytes is 40 to 70% and is about the same for frozen-thawed whole

blood and frozen-thawed lymphocytes isolated by a standard Ficoll-Hypaque gradient technique.[8] However, viability of frozen-thawed isolated lymphocytes (115% of the nonfrozen control ± 3% standard error) appears to be somewhat better than that of lymphocytes from frozen-thawed whole blood (66.5% ± 7.5). This observation may, however, be an artifact. Nonfrozen whole blood cultures normally give a better stimulation response than do corresponding isolated lymphocyte cultures. This is probably due to some growth factor in the whole blood that is lost during lymphocyte isolation by the Ficoll-Hypaque gradient technique. The freeze-thaw procedure described above for whole blood enriches for lymphocytes and involves a washing procedure, which probably also eliminates the "growth factor." Thus, the lymphocytes isolated in this way would no longer give the whole blood stimulation response, but would behave rather more like gradient-isolated lymphocytes. This was found to be the case. Viabilities for frozen-thawed whole blood samples were the same as corresponding nonfrozen gradient-isolated lymphocytes. Thus, the freeze-thaw procedure itself does not appear to affect lymphocyte viability.

The final category of improvements in the existing flow cytometric technique is in data collection and handling. The system for transfer of data from the flow cytometer data storage device to permanent storage has been converted from a PDP-11/45 housed in a separate room from the flow cytometer to a compact LSI-11 computer housed in the same room as the flow cytometer. The data storage devices now used are small inexpensive floppy disks rather than the formerly used large computer disks. These improvements were carried out by Gary Salzman of LS-2, and have made data transfer much easier and data storage much more compact. In addition, Mark Wilder of LS-4 has written a new program for the LSI-11 that allows for display of a linear or semilogarithmic overlay plot of the control and experimental data immediately after transfer of data from the flow cytometer data storage devices. This has greatly improved data analyses during the course of experiments.

Progress toward standardization of data handling among workers on this project has been made. In the progress report October 1, 1977 - September 30, 1976,[6] we pointed out the advantages of expressing results as the stimulation difference (S.D.) instead of the stimulation ratio (S.R.) (see Table I, footnotes c and d for calculation of S.D. and S.R.). Workers at the University of Minnesota independently reached the same conclusion about treatment of their data. This point was discussed and agreed upon at the last project review meeting on September 13, 1979, at NVSL in Ames, Iowa. We suggested also that the stimulation difference be expressed as a percent rather than as raw counts of radioactivity (Minnesota) or raw cell counts (LASL & NVSL) so that we could directly compare data from the three laboratories. Discussions will continue about the most convenient and reliable way of expressing results.

B. Field Study Using the Present Flow Cytometic Technique

Animals from a cattle herd suspected of harboring bovine TB (Randall herd located in Canton, Texas) were bled from the jugular vein once per week for 7 weeks starting January 22, 1979 with an additional bleeding (making a total of eight bleedings) done at 3.5 weeks, immediately after reading the caudal fold tuberculin test (skin test). Samples were sent to LASL and NVSL for analysis by the flow cytometric technique for detection of antigen-induced lymphocyte stimulation and to Minnesota for comparable analysis by incorporation of ^{3}H-thymidine into DNA (a measurement of DNA synthesis). At the beginning of the study the herd consisted of 20 cows, 1 bull, and 3 calves. During the course of the study, 7 more calves were born, making a total of 31 animals.

Table I gives the compiled lymphocyte stimulation (L.S.) results from LASL expressed as both S.D. and S.R. for all bleedings. No data were obtained for some samples because of microbial contamination of the culture or general poor quality of the sample. For bleeding 2, only 10 samples were cultured. Samples from this bleeding were 2 days in transit from Texas to LASL instead of the usual 1 day, and, from previous experience,

TABLE I

LASL LYMPHOCYTE STIMULATION RESULTS - RANDALL HERD

Animal No.	Bleeding 1 (0 week) S.R.[c]	Bleeding 1 (0 week) S.D.(%)[d]	Bleeding 2 (1 week) S.R.	Bleeding 2 (1 week) S.D.(%)	Bleeding 3 (2 weeks) S.R.	Bleeding 3 (2 weeks) S.D.(%)	Skin Test After No. 4 / Bleeding 4 (3 weeks) S.R.	Bleeding 4 (3 weeks) S.D.(%)	Read Skin Test After No. 5 / Bleeding 5 (3.4 weeks) S.R.	Bleeding 5 (3.4 weeks) S.D.(%)	Bleeding 6 (4 weeks) S.R.	Bleeding 6 (4 weeks) S.D.(%)	Bleeding 7 (5 weeks) S.R.	Bleeding 7 (5 weeks) S.D.(%)	Bleeding 8[a] (6 weeks) S.R.	Bleeding 8[a] (6 weeks) S.D.(%)	Skin Test (S.T.)	Lymph Stim.[b] (L.S.)
74ADU9367	1.8	4	N.D.[f]		1.1	1	0.9	0	1.7	3	1.1	1	1.5	2	2.0	2	-	-
68	1.1	2	4.1	16	N.D.		1.8	10	1.5	4	2.1[e]	7[e]	1.4	2	1.4	4	-	±
69	1.3	3	N.D.		1.1	1	1.1	1	1.9	7	N.R.[g]		1.8	4	0.9	0	-	-
70	2.0	8	6.9	24	1.7	7	3.3	19	3.6[e]	25[e]	N.R.		6.6[e]	31[e]	0.9[e]	0[e]	+	±
71	1.5	4	0.9	0	1.0	0	2.1	11	1.7	4	2.3	6	1.7	5	1.2	2	-	±
72	2.0	7	N.D.		1.4[e]	2[e]	1.4	5	1.6	4	N.R.		2.1	7	2.3[e]	7[e]	-	-
73	6.6	48	N.D.		1.7[e]	12[e]	2.0	14	5.5	34	N.R.		10.3[e]	57[e]	7.8[e]	57[e]	+	+
74	2.7[e]	21[e]	N.D.		1.3[e]	3[e]	3.2	15	5.3[e]	22[e]	N.R.		5.0[e]	21[e]	3.0	4	+	±
75	1.6	7	1.2	4	1.3	4	1.8	11	N.R.		N.R.		N.R.		1.7	7	+	±
76	1.4	4	N.D.		0.8	0	1.0	0	2.4[e]	5[e]	N.R.		2.2	5	1.3[e]	1[e]	-	-
77	3.6	27	N.D.		1.8	9	1.6	6	2.4[e]	16[e]	4.1[e]	19[e]	6.6[e]	34[e]	13.1[e]	52[e]	+	+
78	1.1	2	N.D.		1.3	4	1.0	0	1.9	5	0.6	0	3.0	8	1.5	3	-	±
79	0.8	0	6.6	33	1.2	3	2.6	14	3.4[e]	18[e]	2.4[e]	10[e]	3.4[e]	16[e]	3.0[e]	15[e]	+	+
80	1.1	0	N.D.		N.D.		0.6	0	N.D.		1.0	0	N.R.		1.4	1	-	-
81	1.6	4	N.R.		1.2	1	0.7	0	2.4	5	N.R.		0.8	0	1.4	2	-	-
82	N.D.		1.1	2	0.8	0	1.6	7	5	25[e]	1.0	0	1.1	0	1.0	0	-	±
83	1.6	19	7.8	46	1.6[e]	9[e]	1.9	16	3.2[e]	30[e]	N.R.		7.2[e]	38[e]	N.R.		+	+
84	2.4	26	10.2	40	2.1[e]	8[e]	3.0	12	5.5[e]	27[e]	9.0	39	6.6[e]	31[e]	7.9[e]	42[e]	+	+
85	3.3	41	N.D.		5.7	33	3.0	16	7.0[e]	34[e]	7.9[e]	41[e]	15.2[e]	56[e]	8.5[e]	48[e]	+	+
86	1.3	4	N.D.		0.8	0	2.6	14	2.1	7	5.0[e]	20[e]	N.R.		5.0[e]	28[e]	+	±
(calf) 87	1.4	6	2.2	6	N.R.		1.2	2	5.8	22	N.R.		1.2	0	0.8	0	-	±
(calf) 88	1.7	11	N.D.		1.3	3	1.1	1	1.3	3	N.R.		1.5	3	1.1	0	-	±
(calf) 89	1.0	1	N.R.		1.0	0	1.1	1	1.3	2	N.R.		1.6	2	1.1	0	-	-
" 9167	N.R.		N.D.		1.0	0	1.5	7	2.1	4	N.R.		1.3	2	1.8	2	-	-
" 9186	N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		0.9	0	-	-
" 9187	N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		1.4	10	-	-
" 9188	N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.D.		-	-
" 9436	N.R.		N.R.		1.4	3	1.0	0	2.4	7	N.R.		N.D.		1.6	6	-	-
" 9437	N.R.		N.R.		N.R.		1.0	0	1.5	4	1.3	2	1.5	2	N.D.		-	-
" 9438	N.R.		N.R.		1.1	1	0.8	0	2.0	6	N.R.		0.9	0	0.9	0	-	-
74ACU5196 (bull)	1.2	3	1.2	4	0.9	0	0.9	0	1.8	8	N.R.		1.2	3	0.7	0	-	-

[a] Slaughter bleeding

[b] ±, Part of bleedings positive, part negative. Postive S.R. > 3.0; Questionable S.R. = 2.5-3.0, Negative S.R. < 3.0; Positive S.D. > 8%; Questionable S.D. 5-8%; Negative S.D. < 5%.

[c] S.R. - Stimulation ratio calculated as follows:

$$\frac{\text{\% stimulated lymphocytes (antigen-treated)}}{\text{\% stimulated lymphocytes (control)}}$$

[d] S. D. - Stimulation difference calculated as follows:[% stimulated lymphocytes (antigen-treated)] - [% stimulated lymphocytes (control)].

[e] Average of two determinations (i.e., two antigen-treated and two control cultures).

[f] N.D. - No data because of contaminated or poor quality of sample. There was no data from a number of the samples from bleeding 2 because the blood was delayed 2 days in transit and thus, only a few samples were cultured.

we suspected that little useful data would be obtained from testing them. Unexpectedly, the 10 samples cultured did yield useful results. In a number of cases, samples for particular animals were not received. Sometimes there was difficulty in bringing certain animals up for bleeding. Also, there were several calves born during the course of the study and, thus, samples for all bleedings of these animals could not be received. At bleeding 6, only 12 samples were received at LASL. The reason for this is unknown. The last two columns of Table I contain the results of the skin test and a qualitative average of the multiple L.S. tests.

A question in the field study was whetner the L.S. test would detect all of the diseased animals. Table II shows the response of the 10 skin test reactors to the lymphocyte stimulation test. Results are expressed as percent of bleedings for which the animals were L.S. positive, negative, or questionable. Limits for these classifications are defined in Table I, footnote b. Forty percent of the reactors gave positive L.S. tests at 100% of the bleedings. Only one animal, 75, gave a positive L.S. response at less than 50% of the bleedings. This animal was bled only five times and had recently calved at the time of slaughter. Variations in the magnitude of the L.S. response from a single animal suggest that the immunological state, hence, test results (L.S. and skin test) of a single animal may vary with time. It should be noted (Table II, column 5) that all skin test reactors, except animal 77 which was questionable, showed a positive L.S. test at bleeding 4 (3 wks). Bleeding 4 was just prior to the skin test and, thus, is probably the only valid comparison of the L.S. test to the skin test. Results from bacterial cultures for the causative agent, Mycobacterium bovis, and histopathology results (Table II, columns 6 and 7) agree well with skin test and L.S. data. Note that in all cases but one, instances of negative culture and histopathology data correspond to animals that did not test L.S. positive 100% of the time.

We were also interested in the reproducibility of the L.S. test. Figure 2 shows stimulation data for some of the skin test positive animals plotted as a function of time. These graphs illustrate that the S. D., although it varied for a single

TABLE II

LASL LYMPHOCYTE STIMULATION
RESULTS[a] ON SKIN TEST POSITIVE ANIMALS

Animal No.	Percent of Total Bleedings			Results At Bleeding 4	Bacterial[b] Cultures	Lung[c] Lesions
	Positive	Questionable	Negative			
74ADU9373	100	0	0	+	–	+
83	100	0	0	+	+	+
84	100	0	0	+	+	+
85	100	0	0	+	+	Slight
77	86	14	0	±	+	–
70	62	25	13	+	+	+
74	67	33	0	+	–	–
86	50	17	33	+	–	+
75[d]	20	0	80	+	+	+
79	75	0	25	+	+	+

[a] See Table I, footnote, b, for explanation of positive, questionable, and negative.

[b] Positive response – M. bovis detected.
Negative response – M. bovis not detected.

[c] Detected at slaughter.

[d] Only 5 bleedings received.

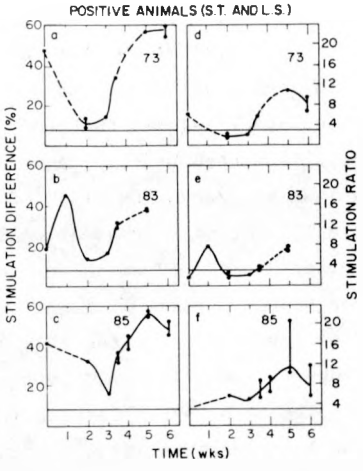

Fig. 2. Lymphocyte stimulation as a function of time (wks) for six animals that were skin test positive at 3.5 weeks and were lymphocyte stimulation (L.S.) positive at all bleedings. Methods for calculating S.D. and S.R. are described in Table I, footnotes b and c, respectively. Each panel is data for all bleedings of one animal. Only the animal number digits which are unique to each animal, are given. The dashed lines indicate no data for one or more bleedings. The solid horizonal lines represent the lower threshold for a positive response. Where determinations on duplicate cultures were done, both data points are plotted.

animal during the study, stayed at or above the positive threshold (solid horizontal line) throughout the study. On the other hand, the positive threshold using the S.R. was somewhat harder to define. Another observation made when comparing the S. D. and the S.R. is that the variability of the S.R. for a single animal for any particular time point is much greater than that of the S.D. This is particularly evident in a comparison of Fig. 2c (S.D., 5 weeks) and the Fig. 2f (S.R., 5 weeks) for animal 85. In addition, the manner in which the graphs are plotted is slightly misleading when comparing the percent variation in replicate samples for the two methods of expressing data. The S.D. scale on the ordinate is much more expanded than the S.R. ordinate scale. Therefore, what looks like a small variation in both the S.R. and S.D. is in fact a much larger variation in the S.R. For example, for animal 83 (Fig. 2b, S.D. and Fig. 2e, S.R.), the variation at bleeding 6 (week 5) for S.R. is 13%, whereas, it is only 1.6% for the S.D.

Figure 3 shows plots of S.D. as a function of time for animals that were skin test positive but were L.S. positive at only part of the bleedings. It should be emphasized again that all of these animals were L.S. positive at 3 weeks (bleeding 4) when the skin test was performed (see above), and that this is probably the only time that a valid comparison can be made between the two procedures. One of these animals (74, Fig. 3a) was classified NGL (no gross lesions) and showed negative cultures for M. bovis (Table II).

Figure 4 shows S.D. plotted as a function of time for animals that were transiently L.S. positive but skin test negative. These animals were neither strongly positive nor positive at more than two bleedings. Table III shows L.S. results expressed as percent of bleedings where the transiently positive animals were classified as L.S. positive, negative, or questionable. Note that two of the transiently positive animals, 82 and 87 (Figs. 4c and 4f, respectively), showed a single positive response following the skin test. Figure 5 demonstrates that this phenomenon can also be observed with most of the negative animals, both skin test and L.S., although the L.S. response after skin testing in these animals did not reach the positive threshold.

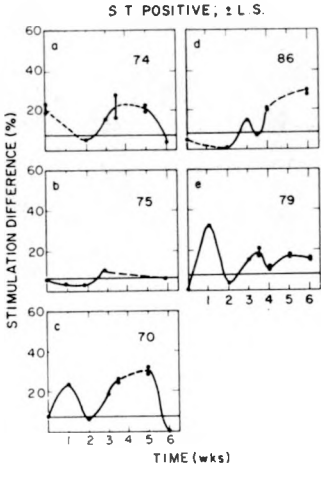

Fig. 3. Lymphocyte stimulation as a function of time (wks) for five animals that were skin test positive at 3.5 wks and were L.S. positive at some of the bleedings. Terms and symbols as in Fig. 1.

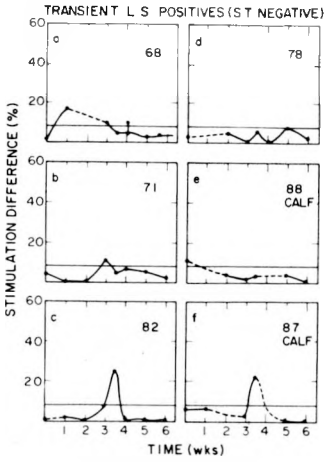

Fig. 4. Lymphocyte stimulation as a function of time (wks) for six animals that were skin test negative at 3.5 wks and were transiently L.S. positive. Terms and symbols as in Fig. 1.

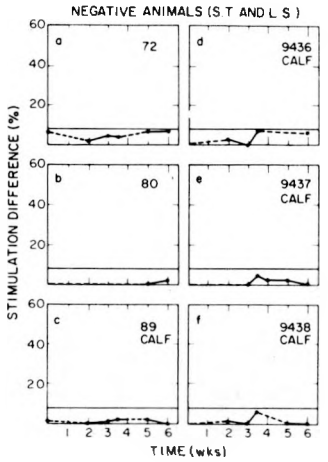

Fig. 5. Lymphocyte stimulation as a function of time (wks) for six animals that were skin test negative at 3.5 wks and L.S. negative at all bleedings. Terms and symbols as in Fig. 1.

TABLE III

LASL RESULTS ON TRANSIENTLY LYMPHOCYTE STIMULATION POSITIVE ANIMALS THAT SKIN TESTED NEGATIVE

Animal No.	Percent of Total Bleedings		
	Positive[a]	Questionable[a]	Negative[a]
74ADU9368	28	14	58
71	12.5	25	62.5
82	14	14	72
78	14	14	72
(calf) 88	17	0	83
(calf) 87	17	33	50

[a]See Table I, footnote b, for definition of positive, questionable, and negative.

In conclusion, this was a very successful field study. First, the herd had several stongly positive animals, which was not the case with other field studies (e.g., Frasie herd). Next the problems associated with previous field studies were minimized in this study. The blood arrived in good condition with only a moderate number of contaminated or clotted samples. The exceptions to this were bleeding 2, where the blood arrived one day late because of adverse weather conditions, and bleeding 6, where only 12 samples were received.

The L.S. test corresponded very well to results from the skin test, bacterial cultures, and histopathology. All skin test reactors were L.S. positive at the time of the skin test. As explained above, comparison of the lymphocyte stimulation and skin test data is probably only valid at that time. The L.S. response varied in magnitude, but was consistently positive for infected animals. This variation is probably a real phenomenon and represents variation in the immunological state of a particular animal.

C. Testing and Comparison of PPD-B Preparations from Different Sources Preparations of PPD-B from Australia and Weybridge, England were compared to a preparation from NVSL by the flow cytometric lymphocyte stimulation assay. Seven PPD-B-sensitized animals, four negative animals, and one experimentally infected animal were tested at three weekly bleedings. The PPD-B concentration, which gave the optimum lymphocyte stimulation response, was determined to be 13 μg/ml for all three preparations. Table IV shows that there was very little difference in the activity of the three preparations when tested with the flow cytometric assay for lymphocyte stimulation. L.S. results with all three preparations agreed with the known disease state of the animal. Workers at NVSL, using the flow cytometric assay, and at Minnesota, using measurement of DNA synthesis rate as an assay, reached the same conclusions.

TABLE IV

COMPARISON OF PPD SOURCES

Animal No.	Disease State	Australian		Ames		Weybridge	
		*	+	*	+	*	+
182	PPD-B Sensitized	21.7	2.7	23.5	2.9	11.0	1.9
183	PPD-B Sensitized	11.1	1.9	18.0	2.5	2.5	1.2
184	PPD-B Sensitized	41.9	4.7	48.4	5.2	38.2	4.4
186	PPD-B Sensitized	18.5	2.2	22.8	2.4	11.1	1.7
187	PPD-B Sensitized	6.0	1.4	13.0	1.8	5.0	1.3
188	PPD-B Sensitized	32.9	2.7	41.3	3.2	26.4	2.4
189	PPD-B Sensitized	17.5	1.8	23.0	2.0	14.2	1.6
N_1	Negative	1.3	1.2	0.9	1.1	1.2	1.2
N_2	Negative	3.5	1.4	5.8	1.7	0	0
N_4	Negative	8.1	1.7	6.0	1.5	1.4	1.1
8067	Infected with *M. bovis*	34.6	4.3	32.0	4.0	24.0	3.3

*Stimulation difference

+Stimulation ratio

D. A New, Rapid Technique for the Detection of Antigen-Induced (PPD-B) Lymphocyte Stimulation

The flow cytometric technique for assay of antigen-induced lymphocyte stimulation is reliable and reproducible. However, this technique involves a culture time of 6 days before optimum detection of lymphocyte stimulation is possible. Another accepted technique for monitoring stimulation is ^{3}H-thymidine incorporation into DNA (measurement of DNA synthesis), for which a 5 day culture period is required for detection.[9] Thus, it is apparent that there is a need for a test that requires less culture time to facilitate rapid diagnosis of disease in animals.

Some of the earliest events that occur in lymphocytes after antigen stimulation are changes in the cell membrane. Probably the initial event is the binding of antigen to receptors on the cell surface. This induces other membrane changes, in particular increases in the transport of certain ions and nutrients, such as amino acids, glucose, potassium, and calcium.[10] Other cellular changes, such as the onset of DNA synthesis and cell proliferation, occur much later.

Recent results from our laboratory show that we can measure an increase in the uptake rate of a nonmetabolizable* analog of the amino acid alanine (α-aminoisobutyric acid, AIB) after a 14-h PPD-B treatment of lymphocyte cultures from animals sensitized to PPD-B (Fig. 6c) or experimentally infected with M. bovis (Fig. 6e). These animals give positive responses when assayed by either the flow cytometric method or ^{3}H-thymidine incorporation into DNA.

Figure 6 also shows that a small enhancement in amino acid uptake is detectable after 6.5 h of PPD-B treatment (Fig. 6b) but not after only 3.5 h (Fig. 6a). Figure 6f shows that there is no enhancement in amino acid uptake after PPD-B treatment of pooled lymphocyte cultures from two animals that are negative by the flow cytometric or ^{3}H-thymidine incorporation techniques.

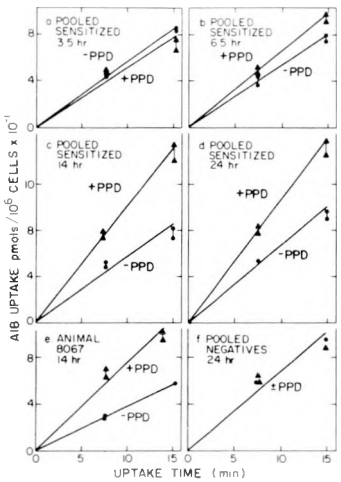

Fig. 6. AIB transport of lymphocytes from pooled sensitized, pooled negative, and experimentally infected animals after various incubation times with PPD-B. Whole blood from five animals sensitized to PPD-B was pooled, and lymphocytes were isolated by the Ficoll-Hypaque gradient technique.[9] Similar lymphocyte isolations were performed with blood from No. 8067, an animal experimentally infected with M. bovis, and blood pooled from two nonsensitized, noninfected animals. The lymphocytes were suspended in RPMI-1640 medium containing 5 to 10% autologous serum. PPD-B at a final concentration of 13.3 μg/ml was added to half of the cultures of each set of animals. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. After various times in PPD-B, samples were harvested by centrifugation and assayed for transport of AIB. Assays were carried out in amino acid-free RPMI-1640 with 5% autologous serum and 500 μM AIB (3 μci/ml ^{3}H -AIB) added. Duplicate aliquots were harvested rapidly by centrifugation at the times indicated on the abcissa, washed rapidly two times in cold RPMI-1640, and the pellets dissolved in 0.1 N NaOH. After neutralization with HCL and addition of scintillation cocktail, the samples were assayed for ^{3}H in a Packard Tri-Carb scintillation counter. Uptake is expressed as pmols of AIB per 10^{6} cells. The error bars represent the range of values obtained with duplicate samples. a)-d), pooled sensitized animals, after PPD-B treatment times of a) 3.5 h, b) 6.5 h, c) 14 h, d) 24 h, e) experimentally infected animal No. 8067 after 14 h PPD-B treatment, f) pooled negative animals after 24 h PPD-B treatment. ●-●, Non PPD-B-treated control; ▲-▲ PPD-B stimulated.

*A nonmetabolizable analog of alanine was used to insure measurement of PPD-B-induced changes in transport only and not changes in protein synthesis.

Figure 7 shows initial rates of AIB uptake, calculated from the slopes of the lines in Fig. 6 a-d, plotted as a function of time of incubation with PPD-B. The enhancement response seems to reach a maximum and plateau by 14 h. The amino acid transport rate in non-PPD-B-treated cultures from sensitized animals stays constant for 14 h but appears to increase slightly at longer times. Additional experiments are necessary for an absolute determination of the best time to measure the enhancement.

Our new technique for detection of lymphocyte stimulation not only provides for a substantial decrease in the culture time required to detect a response (6 days with the flow cytometric assay to 14 h with the amino acid transport enhancement assay), but it also seems to be at least as reproducible, if not more reproducible, from week to week than is the flow cytometric assay. The experimental animal No. 8067 has been tested five times over a period of 3.5 months using the new amino acid transport enhancement technique. Using a 16- to 24-h PPD-B treatment in five experiments, the enhancement ratios* of amino acid transport ranged between 1.75 and 2.2 (average = 1.94 $\pm$ 0.08, standard error). In addition, the pooled sensitized animals used for the experiment described in Figs. 6 and 7 gave comparable enhancement ratios of 1.7 to 1.8. To date, we have examined transport stimulation in lymphocyte cultures from six TB-positive and four TB-negative animals, and in all cases the results were in agreement with the flow cytometric and thymidine incorporation assays, and the known immunologic state of the animals.

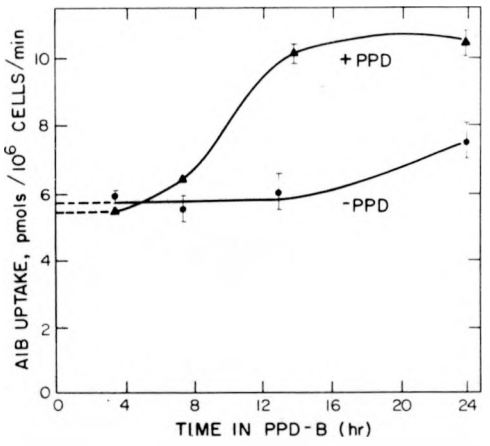

Fig. 7. Initial AIB transport rate as a function of treatment time with PPD-B for pooled sensitized animals. AIB transport rates are expressed as pmols AIB per 10^6 cells per min and were calculated from the slopes of the lines in Fig. 6, a-d. The error bars represent the range of values obtained with duplicate samples. ●-●, non PPD treated control; ▲-▲ PPD-B stimulated.

IV. FUTURE ACTIVITIES

In the last year, we have begun development of a potentially useful technique for the rapid diagnosis of TB in cattle. However, there are many questions remaining to be answered before such a technique could be transferred to the USDA for use as a screening test. In the coming year, we plan to (1) determine the earliest time possible for detection of the PPD-B-induced enhancement of amino acid transport, (2) work on simplification of the present amino acid transport assay, (3) characterize further the enhancement response, (4) investigate the use of a fluorescent amino acid analog in the new assay, (5) screen the new assay for accuracy and reproducibility in a field study of a TB-infected herd (if a suitable herd is available), and (6) investigate the use of a fluorescent membrane potential probe for monitoring early membrane changes after lymphocyte stimulation.

First, it is very important to determine the earliest possible detection time for the enhanced amino acid uptake response. It may be possible and even desirable to detect the response at a PPD-B treatment time of less than 14 h. The advantages of minimizing the time required for the assay were discussed above.

Another effort related to the new technique will be simplification of the amino acid uptake procedure. With the present technique, lymphocytes are isolated from whole blood prior to amino acid transport assays. However, it may be possible to eliminate the lymphocyte isolation step and assay whole blood directly. Red blood cells may not interfere with the assays, because it has been shown that most red blood cells do not take up as much AIB as lymphocytes.[11,12]

*$\frac{\text{transport rate, PPD-B treatment}}{\text{transport rate, no PPD-B treatment}}$

To gain understanding of the cellular events that occur during lymphocyte stimulation, it is necessary to further characterize the enhanced amino acid uptake response to PPD-B. For example, competition studies with other amino acids should yield information about which of the four amino acid transport systems currently recognized in mammalian cells is enhanced by PPD-B. Knowledge in this area will help us to choose other useful probes of the antigen-enhanced transport system.

The radioactive tracer molecule (^{3}H-AIB) presently used is adequate for the characterization studies described above. However, studies with this tracer yield limited information because the measurements represent the average amino acid transport of the total population. Only a very small percentage of the total lymphocyte population undergoes cellular changes in response to an antigen.[10] For a small number of stimulated cells to produce as large an enhancement of the culture-averaged transport as we measure, there must be a very large increase of the transport rate in the responding cells. For example, if only 5% of the total lymphocytes are responders, their amino acid uptake must be enhanced 20-fold to produce a 2-fold increase in the average transport over the total population. It would be very useful, therefore, to be able to study only the responding cells. The flow cytometry instrumentation at LASL could make this possible. We intend to search for a fluorescent amino acid analog that is transported by the PPD-B-stimulated system. It should then be possible to analyze the transport rate of individual lymphocytes, and, thus, quantitate the responding cells, perhaps much earlier than the enhanced transport is evident in the culture as a whole. Such a probe might also be used in viable flow cytometry on the LASL cell sorter to isolate the antigen-stimulated lymphocyte population for further characterization. The enhancement response may be detectable at a much earlier time than 14 h using the single cell analysis technique, which has the capability of detecting small numbers of cells that are more highly fluorescent than the remainder of the population.

A possible problem that might develop in using the fluorescent amino acid analog is that it might not be taken up by the cell in the same manner as the nonfluorescent amino acid. This complication might arise simply because the fluorescent amino acid is much larger than its nonfluorescent counterpart. An alternative way to detect antigen stimulation of lymphocytes might be by measuring changes in the electric potential of the membrane. This potential is often a good indicator of membrane function and has been shown to change significantly with the proliferative state of the cell.[13,14] Fluorescent probes for the measurement of changes in membrane potential are currently available[15] and are well-suited for use in LASL flow cytometry systems. They present the same possibilities for early detection and cell sorting as mentioned above for the fluorescent amino acid analogs.

Finally, it is essential that new disease detection techniques be tested for accuracy and reproducibility in a field test of a herd infected with the disease in question. It would be desirable, therefore, to do a field test using a TB-infected herd in which we would compare our newly developed amino acid transport assay for lymphocyte stimulation by PPD-B to our presently used flow cytometric assay.

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