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Lymphocyte Blastogenesis in the Diagnosis of Tuberculosis in Cattle

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Introduction

Considerable progress has been made towards the eradication of tuberculosis in cattle in the United States;[1] yet, no reliable blood test has been developed for detecting infected animals. An *in vitro* test for tuberculosis must have a high level of sensitivity with adequate specificity to differentiate infections due to *Mycobacterium bovis* and those caused by other mycobacteria. Moreover, it would be desirable that the test system be simple and readily automated. The development of a suitable blood test could provide considerable savings in the cost of field investigations since animals would only be handled once, instead of twice at three day intervals as is currently required in tuberculin skin testing.

Numerous serologic procedures have been evaluated for detecting tuberculous cattle; however, because of limited accuracy, these tests have not come into practical use.[5] More recently lymphocyte immunostimulation tests which are considered to be *in vitro* correlates of delayed-type tuberculin skin hypersensitivity have been investigated. Preliminary results in cattle experimentally-infected with *M. bovis* suggest these procedures may be of value in the diagnosis of tuberculosis.[2,3]

In this report, two *in vitro* procedures for measuring lymphocyte blastogenesis will be discussed. In the first system, cell stimulation is measured by the incorporation of radioactively labeled thymidine in a scintillation counter. In the second system, propidium iodide, a fluorescent dye is used to stain the desoxyribonucleic acid (DNA) of cells; the amount of dye is measured by a flow microfluorometry.

The results of tests conducted on tuberculin positive cows originating from or exposed to animals in herds where tuberculosis was diagnosed will be presented. A comparison of *in vitro* and tuberculin skin tests responses will be made for cattle from which *M. bovis* was isolated.

Materials and Methods

An outline of the protocol for processing blood samples for lymphocyte immunostimulation tests is shown in Figure 1. Details of the methods used for measuring the incorporation of tridiated thymidine into cellular DNA has been previously described.[2] A Packard Tricarb scintillation counter Model No. 3003 was used for the analyses. The stimulation index for cultures of lymphocytes labelled with tridiated thymidine was determined by dividing the value for mitogen stimulated cells by the value for the control culture.

Procedures for staining the nucleic acids of cells with propidium iodide (PI) and the method for cytofluographic analyses of the cells have been reported. A Biophysics Cytofluorograph Model No. 4800A was used for the analyses. The stimulation values for the propidium iodide stained cells was calculated by dividing the number of stimulated cells in the G_2 peak by the number of control cells in the same region (Figure 2).

Tuberculin skin tests in cattle were conducted by injecting 0.1 ml. mammalian old tuberculin in the caudal fold. Bacteriologic examinations of tissues were conducted using 2% sodium hydroxide.[6] Isolates were identified by biochemical and drug susceptibility tests.[5] The *M. bovis* and *M. avium* PPD tuberculins used in lymphocyte cultures were prepared

by ammonium sulfate precipitation of a culture filtrate of M. bovis strain AN-4 and of a culture filtrate of M. avium D-4.*

Results

A comparison of the in vivo and in vitro tests of 10 tuberculin positive cows in an M. bovis infected herd are shown (Table 1). Positive in vitro responses to Bovine PPD were obtained on 8 of the 10 cows. Two cows (Numbers 8 and 10) which had grossly visible lesions on necropsy failed to develop positive responses (stimulation ratio less than 2) to Bovine PPD. In 9 of the 10 animals the lymphocytes stimulation was greater using the homologous M. bovis PPD than using the heterologous M. avium PPD. Cow Number 8 showed minimal responses to both tuberculins.

The results of lymphocyte immunostimulation tests on six tuberculin positive cows and on two tuberculin negative cows before and after tuberculin skin tests are shown (Table 2). Four of six tuberculin positive cows had positive responses to M. bovis PPD on in vitro lymphocyte tests made before tuberculin injection. The 2 tuberculin positive cows (Numbers 67 and 68) which failed to develop positive in vitro responses to M. bovis PPD on the first test did produce positive responses on the second in vitro lymphocyte test conducted 14 days following the first test. Two cows positive on the first test (Numbers 69 and 70) did not develop significant responses to M. bovis PPD on the second test. The two tuberculin negative cows did not develop positive responses on lymphocyte stimulation tests.

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*Prepared by R. D. Angus, Veterinary Services Laboratories, Ames, Iowa.

The *in vitro* blastogenic responses of lymphocytes from 6 tuberculin positive cows and 5 control cows as measured by flow microfluoremetry are shown in Table 3. All 6 cows which developed a positive response to tuberculin produced stimulation ratios to *M. bovis* PPD greater than 2. No responses greater than 1.8 were observed for the 5 control cows. Animal Number 3352 which had the smallest tuberculin skin response developed the greatest stimulation response *in vitro* to *M. bovis* PPD.

Discussion

The *in vitro* lymphocyte immunostimulation procedures described herein provide a potential for earlier detection of *M. bovis* infected animals.[2,3,4] Currently tuberculin retests can be conducted at 60 day intervals; however, for *in vitro* tests, blood could be collected at 7 to 14 day intervals. The *in vitro* system permits testing with several antigens without altering the host response. The use of a blood sample eliminates the need for returning to premises to observe tuberculin skin tests.

Some disadvantages of *in vitro* methods described include the purchasing of expensive equipment, the rapid shipment of blood samples (within 24 hours) to a central laboratory for testing. The use of radioactively labelled compounds and their disposal may be of some concern. It is important to emphasize that sophisticated equipment used for measuring lymphocyte blastogenesis may have mechanical failures resulting in delays in reporting test results.

Additional studies are needed on anergic animals to determine methods for potentiating immunologic responsiveness. Moreover, tests are needed to elucidate the effect of repeated tuberculin skin tests on *in vitro* responses in tuberculous and in noninfected cattle. Investigations are needed to determine the importance of cross-reactions caused by *M. paratuberculosis*, *M. avium* and certain rapidly growing mycobacteria. Information is needed on the significance of skin lesions in altering lymphocyte immunostimulation responses in cattle.

Figure 1. Procedure for culturing bovine white blood cells for immunostimulation tests.

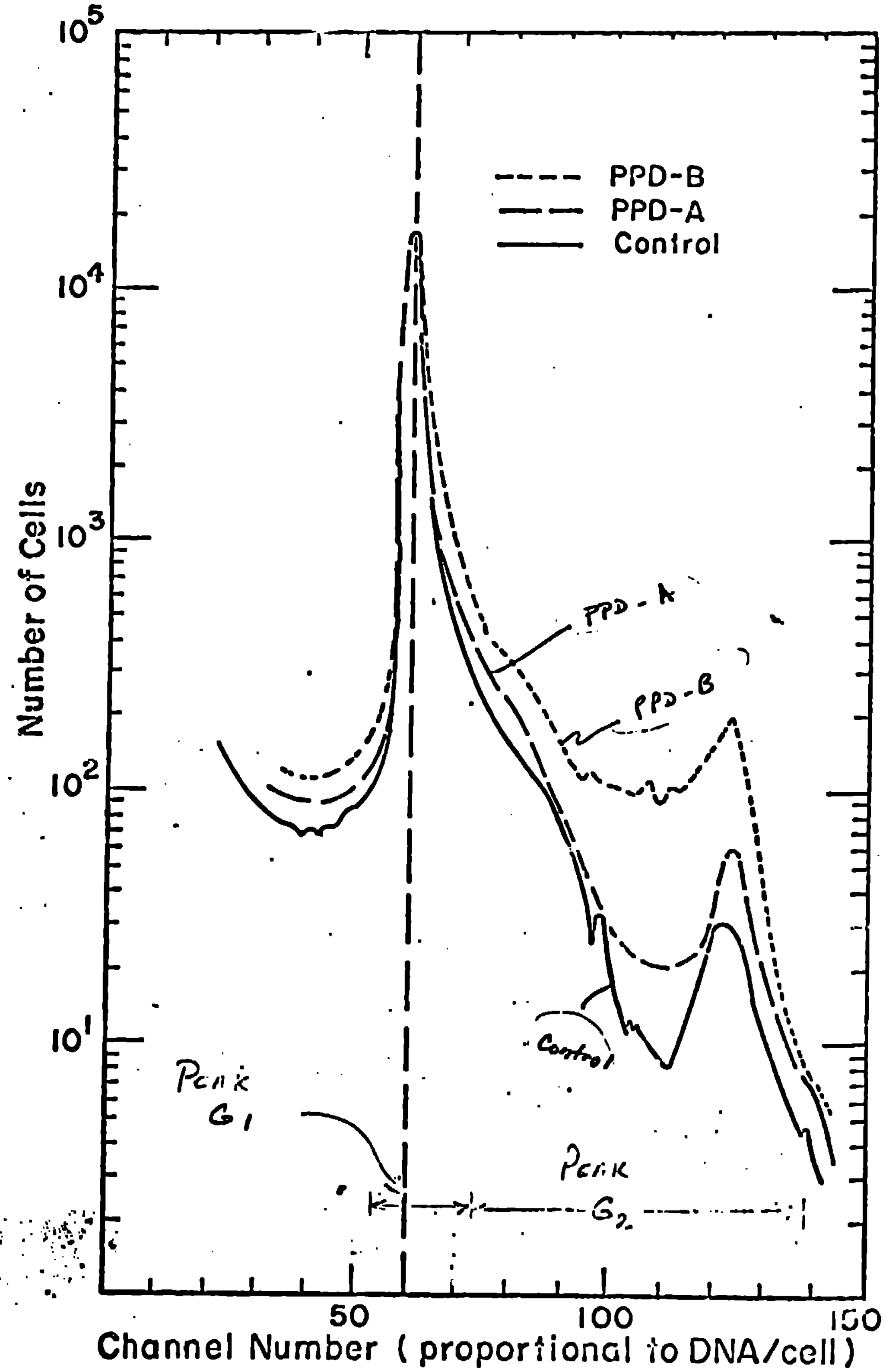

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Figure 2. Semilog display of the DNA fluorescence distribution from control and stimulated bovine lymphocytes stained with propidium iodide.

Table 1. Comparison of *in vivo* and *in vitro* tests in 10 tuberculin positive cows in an *Mycobacterium bovis* infected herd.

Number	Skin Test	Lymphocyte Tests	
		Bov	Av
2	14.5	7.8	3.1
3	20	6.8	1.7
6	10	2.6	1.5
7	34	7.7	1.8
8*	9	1.0	1.0
10*	17	1.8	1.1
12	17.5	3.5	1.6
13	18	19.1	3.1
14	10	3.1	1.5
16	19	2.9	1.4

* Gross lesions present

Table 2. Lymphocyte immunostimulation responses on 8 cows before and after tuberculin skin test.

Animal No.	Skin Test	Lymphocyte Tests			
		Test 1		Test 2	
		Bov	Av	Bov	Av
72	17.6	5.7	1.2	NT	NT
71(+)	28	6.5	1.8	NT	12.9
70(+)	26	9.0	1.6	2.1	2.2
69	9	6.0	1.0	1.3	1.6
68	10	1.3	0.3	4.9	3.2
67(+)	14	1.7	0.2	3.4	1.5
C-1	0	NT	NT	0.4	1.0
C-2	0	NT	NT	0.3	0.7

(+) = M. bovis isolated

NT = Not Tested

Table 3. Lymphocyte blastogenic responses as measured by flow microfluorometry of 6 tuberculin positive cows and 5 control cows.

Animal No.	Lymphocyte Responses to PPD Tuberculin M. bovis	M. avium	Skin Test* Response
3353	3.0	1.26	X_2
3352	5.0	3.3	X_1
3351	2.2	1.5	P_5
2007	2.0	1.5	P_4
2004	3.4	2.8	P_3
2003	3.2	2.2	X_1
5791	1.3	1.1	Negative
6219	1.8	1.4	Negative
6276	1.4	1.1	Negative
5758	1.1	1.0	Negative
6287	1.6	1.3	Negative

*Interdermal skin test made using 0.1 ml. USDA Mammalian Old Tuberculin.

X- diffuse
P - "pea" size

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