BNL 35968

AN OVINE MODEL FOR STUDYING PULMONARY IMMUNE RESPONSES[1,2]

BNL--35968

DE85 007281

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[1] Work supported by U.S. Department of Energy under contract DE-AC02-76CH00016 and by U.S. Public Health Service Grant HL 30865-01. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or allow others to do so, for U.S. Government purposes.

[2] Research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Running Title: Pulmonary Immune Responses.

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ABSTRACT

Anatomical features of the sheep lung make it an excellent model for studying pulmonary immunity. Four specific lung segments were identified which drain exclusively to three separate lymph nodes. One of these segments, the dorsal basal segment of the right lung, is drained by the caudal mediastinal lymph node (CMLN). Cannulation of the efferent lymph duct of the CMLN along with highly localized intrabronchial instillation of antigen provides a functional 'unit' with which to study factors involved in development of pulmonary immune responses. Following intrabronchial immunization there was an increased output of lymphoblasts and specific antibody-forming cells in efferent CMLN lymph. Continuous divergence of efferent lymph eliminated the serum antibody response but did not totally eliminate the appearance of specific antibody in fluid obtained by bronchoalveolar lavage. In these studies localized immunization of the right cranial lobe served as a control. Efferent lymphoblasts produced in response to intrabronchial antigen were labeled with ^{125}I-iododeoxyuridine and their migrational patterns and tissue distribution compared to lymphoblasts obtained from the thoracic duct. The results indicated that pulmonary immunoblasts tend to relocate in lung tissue and reappear with a higher specific activity in pulmonary lymph than in thoracic duct lymph. The reverse was observed with labeled intestinal lymphoblasts.

INTRODUCTION

It has been shown in several species that following the intrabronchial instillation of antigen, specific immune effector lymphocytes can be subsequently demonstrated in lung lavage cell populations and/or the lung parenchyma (1,2,5,14,20,21,23,24,27,35). The origin of these cells is, however, not well documented. There are at least two fundamentally different mechanisms by which the lung may become populated with antigen reactive lymphocytes following intrabronchial immunization. One is the local production of these cells from precursors present in subepithelial lymphoid tissues scattered throughout the respiratory tract. The second mechanism involves the production of effector lymphocytes (or precursors) in regional and/or systemic lymphoid tissues, entrance of these cells into the circulation with subsequent extravasation into the lung.

Previous studies have demonstrated that with respect to the intestine, effector-cell precursors from gut-associated lymphoid tissues enter the circulation primarily via the thoracic duct and randomly repopulate the intestinal lamina propria (8,9,11,12,24,28,29). Their persistence and/or proliferation is, however, thought to be antigen dependent (15-17). These studies rely to great extent on surgical preparations (Thiry-Vella fistulae) which 'functionally' isolate a segment of the gut along with its regional lymphoid system. Cannulation techniques are used to collect cells produced in response to intraluminal antigens and to interrupt their normal entrance into the blood.

The lung like the intestine has a mucosal cell lining which is constantly exposed to environmental materials, including antigens. Lymphoblasts isolated from bronchial lymph nodes (3,4,22,30,31) as well as

pulmonary (19,32) lymph have a propensity to relocate in the lungs rather than the intestine, suggesting that with respect to these two 'mucosal' organs immunoblast distribution is nonrandom and may be dependent upon cell origin. To determine the extent that effector lymphocytes emerging in pulmonary lymph relocate in lung tissue, the distribution of these cells throughout the lung, and the factors that influence their distribution (such as antigen), 'functional' compartmentalization similar to that described above for the gut would be advantageous. In this regard, the anatomical features of the ovine lung may provide an excellent model with which to study the development of pulmonary immune responses.

Pulmonary Lymph Drainage Pathways in Sheep.

The lymph drainage pathways of 20 broncho-pulmonary segments (BPS) were traced following intrabronchial instillation of dyes and/or radiolabeled, killed bacteria. Briefly, 1 to 5 ml of a dye solution (0.2% Evans Blue, 2% Fast Green, 2% Nigrosin) and/or a suspension of E. coli (labeled with ^{125}I-UdR during log-phase growth, heat-killed and washed) were instilled into each BPS with a fiberoptic bronchoscope, wedged and maintained in place for about 20 min. Sheep were euthanized 18-24 hrs later. Localization of the indicator dye to the instilled BPS was confirmed and the regional pulmonary lymph nodes examined for coloration or radioactivity. In addition, the 4 major pulmonary lymph nodes were injected directly with 0.2 ml of dye solution and the efferent lymphatic pathway from each lymph node was traced to its termination in thoracic duct, right lymph duct or the venous sytem.

The critical observation of this study was the identification of 3 BPS-regional lymph node "units" with non-overlapping lymph drainage pathways. Although most BPS were drained by more than one lymph node, the

right cranial, the left apical and the right and left dorsal basal segments were drained exclusively by the cranial bronchial, the caudal bronchial and the caudal mediastinal lymph nodes respectively. Table 1 lists the BPS studied. Table 2 is a summary of the results obtained following dye instillations into the 4 BPS of particular interest. This restricted lymph node drainage pattern was confirmed using ^{125}I-labeled bacteria.

Efferent lymph from the caudal mediastinal lymph node (CMLN) entered the thoracic duct generally via a single duct emerging from the anterior pole of the lymph node. Efferent lymphatics from the caudal bronchial lymph node were found to enter the thoracic duct a point cranial to the juncture of the CMLN. Efferent lymph from the cranial bronchial lymph node entered the blood via the right lymph duct.

Together these results suggest the possibility that with appropriate cannulation procedures, three separate areas of the lung can be 'compartmentalized' i.e. the dorsal basal segments by cannulation of the efferent duct of the CMLN; the left apical segment of the cranial lobe by cannulation of the thoracic duct in the neck region following its ligation near the entrance of efferent duct of the CMLN (cannulation and recirculation would be preferable to prevent opening of new lymph channels due to back-pressure) and; the right cranial segment by cannulation of the right lymph duct.

Immune Responses to Localized Intrabronchial Instillation of Antigen.

To test the compartmentalized lymphatic drainage as it relates to the development of pulmonary immune responses, the efferent duct of the CMLN was cannulated using a modification (6) of the two-stage procedure of Staub et al (33). In these studies two sets, or pairs, of antigens were

used: (a) 0.5-1.0 x 10^{11} horse red blood cells (HRBC) and dog red blood (DRBC) or, (b) 2 x 10^{10} heat-killed E. coli and S. minnesota. With a fiberoptic bronchoscope one antigen was instilled into the right dorsal basal segment while its paired antigen was instilled into the right cranial segment. The bronchoscope remained 'wedged' for 15-20 minutes and upon removal any antigen suspension emerging from the immunized segment was aspirated. A second challenge with the same antigens was given 14 days after the primary challenge. CMLN efferent lymph was quantitatively collected, but none was returned to the animal. Efferent lymph was assayed for specific antibody-forming cells (AFC) using standard hemolytic plaque techniques. To test for AFC against E. coli and S. minn. sheep red blood cells coated with the specific bacterial endotoxin were used in the plaque assay.

Fifty ml of bronchoalveolar lavage (BAL) fluid were obtained from each of the two immunized BPS of the right lung and from a non-immunized segment of the left lung. Prior to assay, BAL fluid was centrifuged and the cell-free supernatant concentrated about 20 fold using Millipore CX-30 immersible filtration units (Millipore Corp., Bedford, MA). Heat-inactivated serum and cell-free BAL fluid were assayed for hemagglutinin activity by mixing 20 µl of 1.5% washed red cell suspension with 20 µl of serially diluted test fluid or serum. Following 1 h of incubation at 37°C, agglutination was determined microscopically.

These experiments are still in progress and only preliminary results are presently available. When heterologous red cells were instilled into the dorsal basal segment of the right lung, specific AFC were first detected in CMLN efferent lymph 4 or 5 days later, reaching a peak on day 6 or 7 postimmunization. Following a second challenge, AFC increased

rapidly with the maximum output being between days 2 and 3. AFC in CMLN efferent lymph were directed exclusively against the red cell antigen instilled in the dorsal basal segment with no AFC activity against red cells used to immunize the right cranial segment.

When heat-killed bacteria were used as antigens the peak out of AFC in pulmonary lymph was earlier, occurring between days 4 and 5. During the early phase of the primary response AFC were primarily directed against the antigen instilled in the right dorsal basal segment, however, a small number of AFC were also detected against red cells coated with the specific endotoxin of bacteria instilled in the right cranial segment. By day 6, AFC in CMLN efferent lymph were directed only against the right dorsal basal antigen. This observation was assumed to be the result of cross-reactivity to antigens shared by the two specific endotoxins and not the consequence of AFC recirculation or direct stimulation of the CMLN by the bacterial antigen instilled in the right cranial segment.

Serum and BAL fluid have been assayed for hemagglutinin activity in 2 sheep which received 2 antigenic (HRBC-DRBC pair) challenges 14 days apart and 1 sheep which had only a primary immunization. In none of the 3 sheep was there a detectable serum antibody response to the antigen instilled in the lung segment (dorsal basal) drained by the CMLN, even after a second immunization. In contrast, serum hemagglutinin activity against red blood cells instilled into the right cranial segment was evident in all animals after primary challenge, with higher titers following the second immunization.

Although the results of antibody assays of fluids obtained from various lung segments by BAL are incomplete, and currently being repeated by more sensitive ELISA techniques, two findings are worthy of mention;

(a) the highest hemagglutinin activity was found in lavage samples from the right cranial segment and was directed against red cells used to immunize that segment. In one sheep, 5 days after the second immunization, the titer in BAL fluid from the right cranial segment was 4-fold higher than the corresponding serum titer; (b) despite of no detectable serum antibody, BAL samples from the right dorsal basal segment contained low hemagglutinin activity against red cells used for immunizing that segment.

Pulmonary Lymphocyte Migration and Tissue Distribution.

The expression of local pulmonary immunity may, to a large extent depend on the migration and tissue distribution patterns of efferent lymph lymphocytes, particularly immunoblasts generated in response to intrabronchial antigens. In view of the current concepts of a common mucosal immune system (4, 22), migrational patterns of pulmonary lymphocytes were compared to those obtained with intestinal (thoracic duct) lymphocytes and, in a limited number of studies, to lymphocytes emergi , in the efferent duct of the prescapular lymph node.

Cannulation of the efferent lymph duct of the CMLN was done using the two-stage procedure of Staub et al. (33) During the same surgical procedure, the thoracic duct (TD) was cannulated just posterior to the entry of the CMLN lymph duct. Cannulation of the efferent lymph duct of the prescapular lymph node was done as a separate surgical procedure a few days later. An indwelling silastic catheter was placed in the external jugular vein and, with expection of collections for labeling and sampling, lymph was continously returned to the blood via a closed system maintained by a special pumping mechanism.

Lymph was collected in sterile, siliconized glass bottles kept at 4°C. Lymphocytes (1 to 21 x 10^9) were concentrated to about 10^8 cells/ml in phosphate-buffered saline containing either 0.5 µCi ^{125}I-iodo-deoxyuridine (^{125}I-UdR New England Nuclear, spec. act. > 2,000 Ci mMol) alone, or ^{125}I-UdR (0.5 µCi) plus 10-20 µCi $Na_2{}^{51}CrO_4$ (New England Nuclear, spc. act. 350-400 mCi/mg) per ml cell suspension and incubated for 45 minutes at 37°C. Cells were washed once, resuspended in about 200 ml of cell-free autologous lymph, and slowly reinfused intravenously or intra-arterially.

To increase the number of lymphoblasts in efferent CMLN lymph, two intrabronchial (dorsal basal segment) instillations of either heterologous red cells or heat-killed bacteria were given, the second instillation being 2 to 3 days prior to cell labeling.

Lymph samples were collected at various times after cell infusion. Cell counts were made using a Coulter Model ZBI and radioactivity of washed, pelleted cells determined using a well-type gamma counter. Counts were corrected for channel spillover (^{51}Cr vs ^{125}I) and expressed as specific activity i.e. cpm per unit number of cells.

To determine the tissue distribution of radioactivity sheep were anesthetized 20-24 hours post cell infusion and the vascular system 'flushed' with 8 liters of saline. Organs were weighed and several representative samples taken for analysis. Each sample was weighed. Radioactivity was determined and expressed as cpm/mg tissue or converted to the percent of activity recovered in various organs.

The results of these studies are summarized in Figures 1-4 and Table 3. When TD (intestinal) lymphoblasts were labeled, cell-associated radioactivity in TD lymph rose rapidly, reaching a peak 9 hours after

infusion (Figure 1a). At this time the specific activity in the TD was about 3 times that found in lung lymph. By 2 days post infusion, radioactivity was barely detectable. In contrast, when pulmonary lymphoblasts were labeled, the specific activity in efferent CMLN lymph was consistently higher than in TD lymph and peaked somewhat later, i.e. day 1, (Figure 1b). Another notable difference was that cell-associated radioactivity was readily detectable in both pulmonary and intestinal lymph for 3 and even 6 days after infusion suggesting that a significant fraction of these may have entered the recirculating pool of lymphocytes.

As shown in Figures 2 and 4a, essentially no difference was observed in the specific activities of efferent CMLN, efferent prescapular lymph and TD lymph following the infusion of ^{51}Cr-labeled TD lymphocytes. When pulmonary lymphocytes were labeled and reinfused, a slight but consistently (all 4 studies) higher specific activity was found in efferent CMLN lymph as compared to TD lymph (Figure 3). As shown in Figure 4b radioactivity in efferent prescapular lymphocytes was similar to that seen in CMLN lymphocytes.

In 3 studies with labeled pulmonary lymphoblasts and 1 study with intestinal (TD) lymphoblasts, lung lavage cell suspensions were obtained 2 or 3 days after cell infusion. Cell-associated radioactivity was consistently detected in BAL samples, however, the amounts were low. Based on the proportion of lung lavaged, it was estimated that on the average less than 0.5% of the ^{125}I-activity injected as labeled pulmonary lymphoblasts was present in the lavagable cell population at the time of sampling. Although the recovery was low, the specific activity (cpm/10^6 lymphocytes) was as high or higher than the specific activity in efferent

pulmonary lymph. In the single study with ^{125}I-labeled TD lymphoblasts, both the estimated recovery (< 0.04%) and the specific activity were low.

The specific activity (cpm/mg tissue) and the percent of recovered radioactivity present in various organs 20 to 24 hours after infusion of labeled lymphoblasts are shown in Table 3. Clearly, ^{125}I-labeled TD lymphoblasts have a marked propensity to localize in the intestine and regional mesenteric lymph nodes. Of the radioactivity recovered, 86% was in the intestinal tract and only 5% was lung associated. In contrast, when ^{125}I-labeled pulmonary immunoblasts were infused, the largest fraction of radioactivity was present in the lung, however, significant amounts of radioiodine were also recovered from the intestinal tract and spleen. Although lymphoblasts were collected only from the antigenically stimulated CMLN, all pulmonary lymph nodes had a specific activity which was several times higher than the specific activity in the mesenteric and prescapular/prefemoral lymph nodes.

Comment

The experimental approaches used in elucidating the development of intestinal immune responses have not been previously adaptable to the lung due both to the lack of access to lymph-borne cells responding to intrabronchial antigens and to the inability to establish separate functional units similar to the Thiry-Vella fistula. Sheep, however, may represent an experimental animal in which such experiments can be done. Cannulation techniques for long-term collection of efferent lymph from the CMLN (6,33) as well as the caudal bronchial lymph node (32) have been described. We have previously shown that intrabronchial administration of antigens into the deep right lung stimulates a marked increase in the output of specific antibody-forming cells (AFC) and lymphoblasts in

efferent lymph of the CMLN (18). Recent evidence suggests these lymph-borne lymphoblasts have a tendency to relocate in the lung (19,32). To further develop the ovine model of pulmonary immunity the current studies were designed to address three specific questions; (a) What are the lymph drainage pathways of various lung segments; (b) Does continuous diversion of efferent CMLN lymph eliminate the systemic response to a highly localized intrabronchial antigenic stimulation and; (c) Do efferent lymph lymphoblasts produced in response to intrabronchial antigens have a propensity to relocate in the lung.

The lymph drainage pathways of 20 broncho-pulmonary segments were traced following intrabronchial instillation of dyes. The results indicated that 3 separate segments of lung were each drained exclusively by a single lymph node, i.e., the right and left dorsal basal segment by the CMLN; the right cranial segment by the cranial bronchial lymph node and; the left apical by the caudal bronchial lymph node. These findings were confirmed using radiolabeled, heat-killed bacteria. This observation suggests that if antigenic stimulation can be confined to the dorsal basal segment, continuous diversion of CMLN lymph would eliminate the systemic component of the immune response (10). The presence of specific antibody and/or specific immune effector cells in this segment of the lung would thus indicate a capacity for the development of local immune responses, independent of the systemic immune apparatus. In contrast to the dorsal basal segment, the immune response to stimulation of the right cranial segment with a second antigen should be unimpaired and serve as a control. Two assumptions are made; (a) particulate antigen does not enter the blood directly nor is it transported to other parts of the lung in sufficient quantity to cause sensitization and, (b) immunologically

reactive lymphocytes leave the node via efferent lymph and do not enter the blood directly. Systemic sensitization can to a degree be tested by determining whether a primary or secondary antibody response is illicited following parenteral immunization.

The results obtained from the dual antigen studies are preliminary. It was clear, however, that AFC in efferent CMLN lymph were directed against the antigen instilled in the dorsal basal segment and that the continuous diversion of CMLN lymph resulted in the absence of detectable serum antibody, even after a second challenge with the same antigen. Serum antibody responses to the antigenic stimulation of the right cranial segment, on the other hand, were vigorous.

Of considerable interest in these experiments was the demonstration of specific antibody in lavages samples obtained from the dorsal basal segment. This finding suggests there is at at least a limited capacity for the generation of AFC locally within the lung. The alternative explanations are that AFC entered the blood directly within the lymph node or that somehow the sheep became systemically sensitized. Further work is required before any firm conclusions can be drawn.

Our results on the tissue distribution of lymphoblasts agree, in general, with those of Hall and co-workers (12, 32). Intestinal blasts accumulated rapidly and almost exclusively in the gut. Pulmonary immunoblasts, on the other hand, tended to localize in the lung although significant ^{125}I-activity was also found in the intestine and spleen. To what extent this is due to lymphoblast "contamination" of efferent CMLN lymph from sources other then lung is unknown. In addition, these studies demonstrated a corresponding difference in the reappearance of radioiodine in the different efferent lymph compartments. Following the infusion of

^{125}I-labeled pulmonary immunoblasts the specific activity in the efferent CMLN lymph was significantly higher than in the thoracic duct; the reverse was observed when intestinal blasts were labeled and reinfused.

A possible explanation of these results is that pulmonary immunoblasts which have entered the blood from the regional CMLN selectively extravasate in the lung. The increase in specific activity in efferent pulmonary lymph may then be due, for the most, to labeled cells which subsequently leave the lung and enter the CMLN via afferent lymphatics. There is no evidence, to our knowledge, that the increase in specific activity would be the result of a selective recicrulation of lymphoblasts directly within the antigenically stimulated lymph node. The hypothesis would also explain the relatively high specific activity of lymphoid cells obtained from the lung air spaces by lavage. If lymphoblasts, on the other hand, enter the lung randomly but are selectively retained, the specific activity in CMLN efferent lymph would be higher following the infusion of intestinal blasts than following the infusion of pulmonary blasts. The problems associated with tissue distribution and migratory patterns of labeled lymphocytes have been critically reviewed by Morris (25).

The influence of antigen in lymphoblast migration and distribution in the lung remains to be clarified. Although immunoblasts used for labeling were obtained only from the antigenically stimulated CMLN, the specific activity in other pulmonary lymph nodes 20-24 hours after infusion was as high or higher as that found in the CMLN. Studies on the role of antigen in the distribution and/or retention of immunoblasts in lung parenchyma are currently in progress using the dual antigen model.

It was somewhat surprising, in view of work published by others (7, 32), that clear differences in the migration of ^{51}Cr-labeled lymphocytes from blood to various lymph compartments were not observed in our studies. ^{51}Cr-labeled TD lymphocytes reappeared in pulmonary and thoracic duct lymph with almost identical specific activities. When pulmonary lymphocytes were labeled and reinfused, a small, but consistently higher specific activity was found in pulmonary lymph as compared to TD lymph. The reason for these differences in results is unclear.

ACKNOWLEDGEMENTS

This work would not have been possible without the excellesnt technical assistance of A. Alberico, K. Conkling, F. Lawson, J. Pedersen, S. Salinas, and T. Weldon.

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FIGURE LEGENDS

Figure 1. Mean (± SE) relative specific activity in thoracic duct lymph (TD) and efferent lymph of the caudal mediastinal lymph (CMLN) after the intravenous infusion of ^{125}I-labeled lymphoblasts obtained from either (a) the thoracic duct or, (b) efferent CMLN lymph. N = 6.

Relative specific activity =

$$\frac{\text{CPM}/10^6 \text{ lymphocytes in lymph sample}}{\text{CPM}/10^6 \text{ lymphocytes in cell infusion}}$$

In these studies 0.9 to 10.7 x 10^9 lymphocytes containing 0.34 to 2.64 x 10^6 cpm radioactivity were infused.

Figure 2. Mean (± SE) relative specific activity in thoracic duct lymph (TD) and efferent lymph of the caudal mediastinal lymph node (CMLN) after the intravenous infusion of thoracic duct lymphocytes labeled *in vitro* with $Na_2{}^{51}CrO_4$. See Figure 2 for calculation of relative specific activity.

In these studies 1.2 to 3.3 x 10^9 lymphocytes containing 1.3 to 8.6 x 10^6 cpm radioactivity were infused.

Figure 3. Mean (± SE) relative activity in thoracic duct lymph (TD) and efferent lymph of the caudal mediastinal lymph node (CMLN) after the intravenous infusion of CMLN efferent lymphocytes labeled *in vitro* with $Na_2{}^{51}CrO_4$. See Figure 2 for calculation of relative specific activity.

In these studies 8.5 to 20.9 lymphocytes containing 5.1 to 27.7 x 10_6 cpm radioactivity.

Figure 4. Specific activity (cpm/10^6 lymphocytes) in thoracic duct lymph (TD), efferent lymph of the caudal mediatinal lymph node

(CMLN), and efferent lymph from the prescapular lymph node (PLN) after the intravenous infusion of either (a) 3.3×10^9 thoracic duct lymphocytes containing 3.1×10^6 CPM ^{51}Cr or; (b) 10.2×10^9 efferent CMLN lymphocytes containing 5.1×10^6 cpm ^{51}Cr.

Table 1. Broncho-pulmonary segments (BPS)[a] instilled with dyes and/or radiolabeled, killed bacteria.

Lobe	BPS Instilled	
	Right Lung	Left Lung
Cranial	Cranial	Apical
(Apical)[b]	Caudal	Cardiac
Middle	Lateral	
(cardiac)	Medial	
Accessory	Dorsal	
(Intermediate)	Ventral	
Caudal		
(Diaphragmatic)	Apical	Apical
	Subapical	Subapical
	Ventral basal	Ventral basal
	Lateral basal	Lateral basal
	Dorsal basal	Dorsal basal
	Medial basal	Medial basal

[a] Obtained from references 13 and 34.

[b] Alternate terminology in parenthesis.

Table 2. Results of intrabronchial administration of dye suspensions into the four broncho-pulmonary segments drained by a single lymph node.

Lobe	Broncho-pulmonary segment	No. of studies	Lymph node stained[a]			
			Caudal Mediastinal		Bronchial	
			Caudal	Cranial	Caudal	Cranial
Right Cranial	Cranial	6	-	-	-	+
Right Caudal	Dorsal Basal	5	+		-	-
Left Cranial	Apical	6	-	-	+	-
Left Caudal	Dorsal Basal	5	+	-	-	-

[a] + = stained, - = not stained.

Table 3 The Mean Specific Activity* and the Mean Percent of the Recovered Radioactivity Associated with Various Tissues 20-24 hours after the Infusion of ^{125}I-Labeled Lymphoblasts

	Lymphoblasts from Efferent Lymph of CMLN (N=6)		Lymphoblasts from The Thoracic Duct (h-4)	
Tissue	Specific Activity	% Recovered	Specific Activity	% Recovered
Intestine	40	28	332	86
Lung	100	45	46	5
Spleen	179	16	205	3
CMLN	357	3	186	1
BLN	377	< 1	159	< 1
MLN	64	2	551	4
PLN	105	< 1	178	< 1

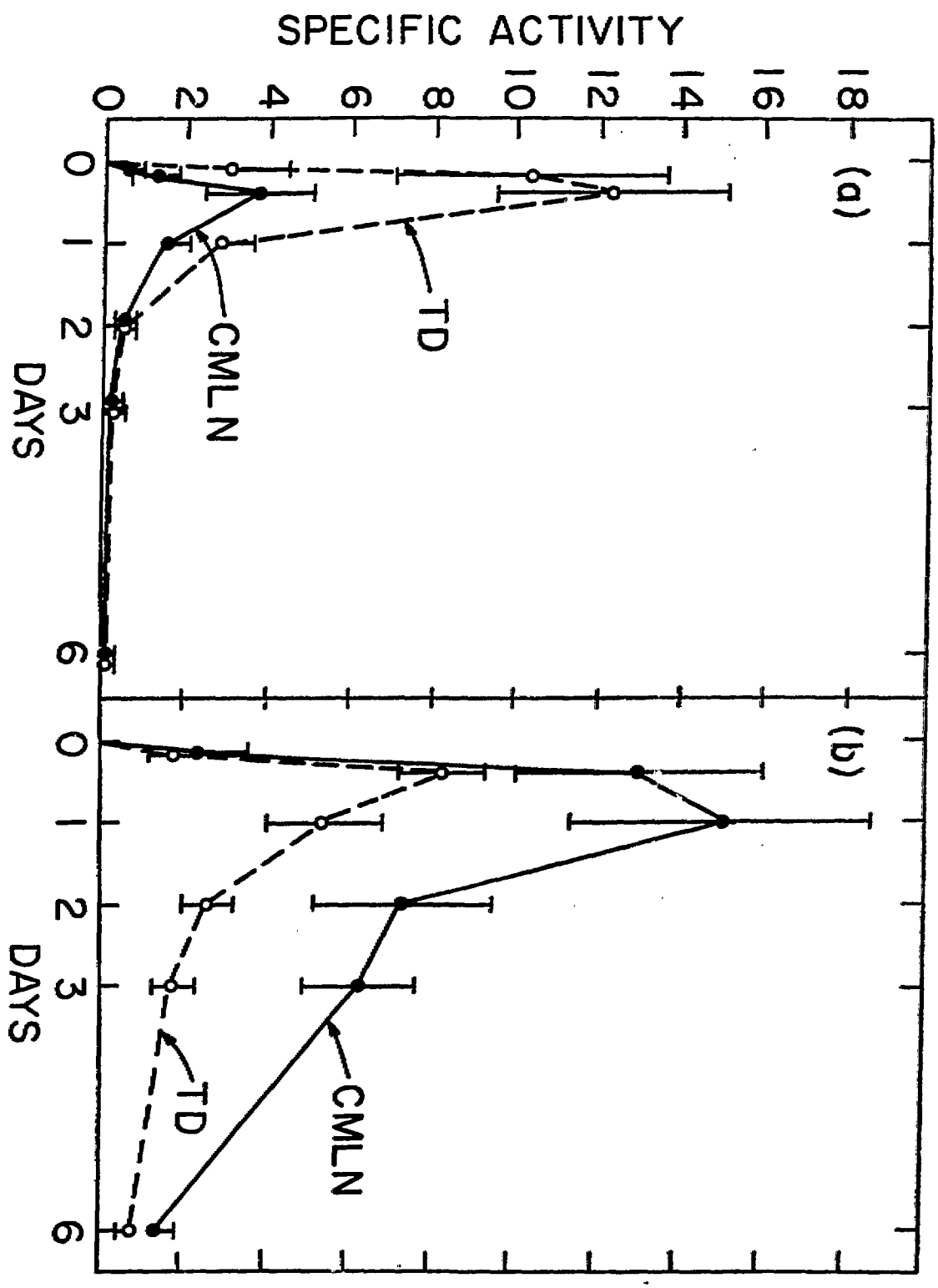
* - activity is expressed as CPM/mg tissue

CMLN - Caudal Mediastinal Lymph Node.

BLN - Bronchial Lymph Node.

MLN - Mesenteric Lymph Node.

PLN - Prefemoral and/or Prescapular Lymph Nodes.



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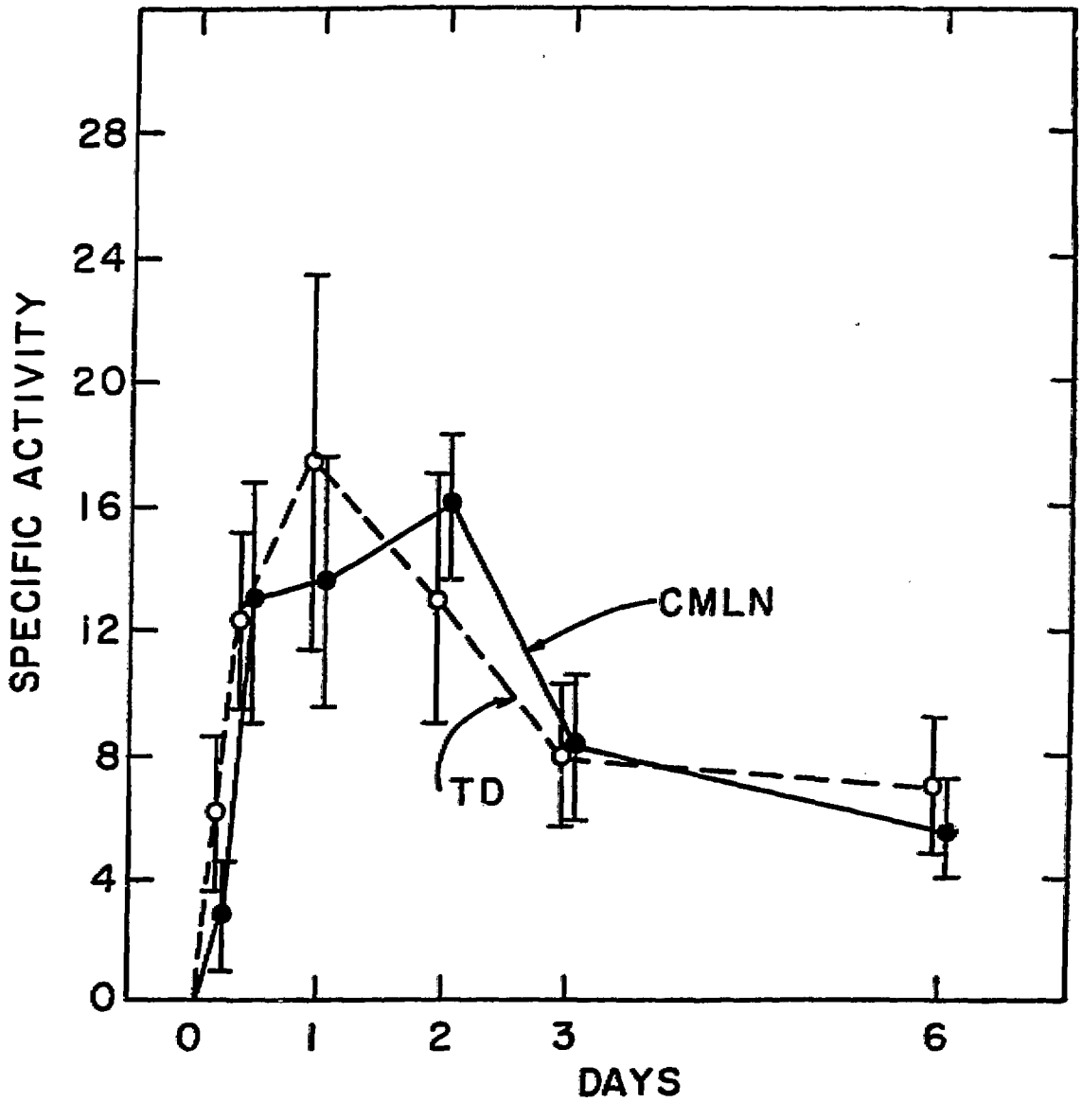

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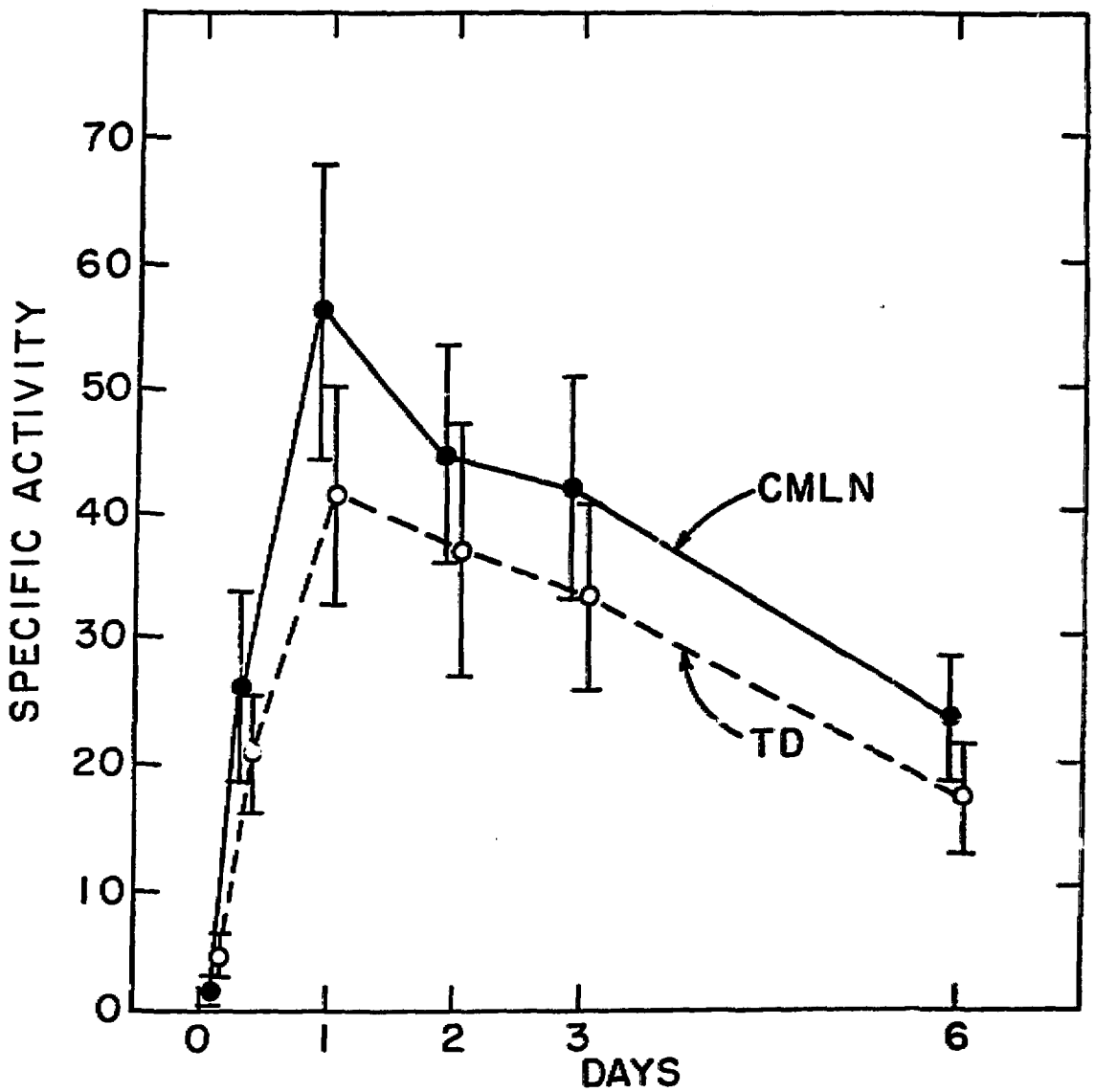

Figure 3. (Neg. No. 11-534-84)