STUDIES ON THE ANTIGENICITY OF LYMPHOMATOUS TISSUE FROM THE DOG

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ABSTRACT

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The object of this study was to determine if lymph node cells from dogs with malignant lymphoma had an antigen or antigens that were not present on normal lymph node cells. The results indicated a change in the antigenic structure of canine lymphomatous lymph node cells. This change was evidenced by the absence of "normal" antigens or by the presence of a unique antigen or antigens on the lymphomatous lymph node cells.

The antigenic changes were detected using normal canine serum antiserum (rabbit origin), lymphomatous canine serum antiserum (rabbit origin), lymphomatous canine lymph node antiserum (rabbit origin) and normal canine lymph node antiserum (rabbit origin) to develop immunoprecipitates with normal canine lymph nodes and lymphomatous canine lymph nodes using immunoelectrophoretic techniques.

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By

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INTRODUCTION

The antigenicity of malignant tissues has been studied with gel diffusion techniques (Björkland, 1956; Cryan et al., 1966; DeCarvalho, 1960; and Korngold, 1957). It has been generally conceded that malignant tissue cells do possess a unique antigenic structure.

McKenna and Prier (1966) were unable to detect a unique antigen in lymphomas from five dogs. However, DeCarvalho (1960) did detect unique antigens on malignant cells from human patients with chronic lymphatic leukemia. Lampkin-Hibbard and McCain (1965) were able to produce antibodies in guinea pigs against lymphomas by using a nucleoprotein extract from malignant lymphoma cells.

The objective of this study was to determine if lymph node cells from dogs with malignant lymphoma had antigens that were not present on normal lymph node cells that could be detected by immunoelectrophoretic techniques.

LITERATURE REVIEW

Malignant lymphoma is one of many neoplasms that invade man and lower animals. Extensive research has been done on the antigenic properties of numerous malignant neoplasms, spontaneously and chemically induced, including the lymphomas. In this review of the literature, only information relating to the antigenic properties of lymphomas and other neoplastic diseases will be included.

Specific tumor antigens have been detected by various investigators. These include Björkland and Paulsson (1962), Cryan et al. (1966), DeCarvalho (1960), Greenspan et al. (1963), Korngold (1957), Korngold and VanLeeuwen (1957), McKenna and Prier (1966), McKenna et al. (1962), McKenna et al. (1964), Strausser and Goldman (1965), Taylor et al. (1959) and Yagi and Pressman (1961).

Foley (1953) induced fibrosarcomas in mice by implanting methylcholanthrene crystals subcutaneously in C3H (Hestar subline) mice, or by subcutaneous injection of 1 mg of methylcholanthrene in 0.05 ml of lard. After the tumors had grown to a sufficient size, they were transplanted to other mice. The same transplantation procedure was used for spontaneously developed mammary carcinomas. After several transplantations, the tumors which developed were ligated and

eventually died. The animals were then challenged with another tumor transplant. The animals that had received the chemically induced fibrosarcoma were immune to an additional transplant. The animals receiving the mammary carcinoma transplants did not develop immunity against additional transplants. Thus, some malignant tissues may contain an antigen or antigens that are not present in normal tissue. Prehn (1960) reported similar results by inducing fibrosarcomas with dibenz (a,h)-anthracene. Old et al. (1962) noted that 3-methylcholanthrene-induced fibrosarcomas were more antigenic than fibrosarcomas induced with 3,4-9,10-dibenzpyrene.

Using hemagglutination techniques, Blakemore and McKenna (1962) reported a serologically distinct G antigen from the HeLa and J111 cell lines. McKenna et al. (1966) tested numerous cell lines from malignant tissues for the presence of the HeLa G antigen. The antigen was present in approximately 50% of the malignant tissue cell lines. The antigen was absent from all normal tissue cell lines except for the Minnesota embryonal esophagus cell line.

Taylor et al. (1959) prepared rabbit antisera against HEp No. 2 tissue culture cells, human sarcoma, germfree (G. F.) chicken tumor, Rous sarcoma and normal G. F. chicken muscle cells. Half of each of the antigen preparations were treated with fluorocarbon to remove any nonviral or host tissue antigens. The use of fluorocarbon to remove normal tissue antigen was first reported by Gessler et al. (1956,

1956a). Antisera against the non-fluorocarbon-treated antigens cross-reacted with each type of tumor cell line antigen and with the normal tissue. Antisera against the fluorocarbon-treated antigens reacted only with their respective antigens.

MacKenzie and Kidd (1945) used complement fixation tests to demonstrate an antigen unique to the Brown-Pearce carcinoma. The antigen was not present in normal rabbit tissues or other neoplastic tissues.

Björkland (1956) pooled tissues from several different carcinomas and several normal tissues. The pools were extracted twice with diethyl ether and the extract injected into a horse. The resulting antisera were adsorbed with human serum to remove any normal tissue antigens. Four different cellular antigens were demonstrated using a modified Ouchterlony gel diffusion technique. The antigens were not tumor specific, however, as they cross-reacted with other tumors and normal tissue even after adsorption.

Using gel diffusion techniques, Cryan et al. (1966), demonstrated specific antigens in spontaneously occurring mammary carcinomas of Swiss Webster mice. These antigens were not present in normal mammary tissue. DeCarvalho (1960) studied the antigenic characteristics of fluorocarbon-purified extracts of carcinomas, sarcomas, acute stem cell leukemia cells and chronic lymphocytic leukemia cells. Antisera against the tissue extracts were prepared in rabbits. The anti-carcinoma and the anti-sarcoma antisera reacted

with both the carcinoma and sarcoma extracts, but not the acute stem cell leukemia or chronic lymphocytic leukemia cell extracts. The anti-acute stem cell leukemia antiserum and the anti-chronic lymphocytic leukemia antiserum reacted only with their respective antigens. This suggested the presence of a distinct antigen for the acute stem cell leukemia cell and for the chronic lymphatic leukemia cell. Carcinoma and sarcoma appeared to have the same or a similar antigen or antigens.

Greenspan et al. (1963) prepared anti-leukemic brain, anti-normal brain and anti-Hodgkin's disease lymph node antisera in human volunteers. The antisera were adsorbed with normal brain tissue. Each antiserum reacted with its own antigen. The anti-leukemic brain antiserum also reacted with Hodgkin's disease lymph nodes, human leukemic reticulo-endothelial cells and leukemic mouse brain.

Lampkin-Hibbard and McCain (1965) extracted the nucleoproteins from various mouse and human lymphomas and prepared antisera against each nucleoprotein. By injecting the antisera into mice, the authors were able to protect the mice from lymphoma transplants.

McKenna and Prier (1966) used canine neoplasms to study neoplastic antigens. Normal tissues were pooled for use in adsorption procedures and in antisera preparation. The authors used several types of malignant neoplasms. Specific antigens were demonstrated in adenocarcinomas, osteosarcomas.

basal cell carcinomas, bile duct carcinomas and squamous cell carcinomas. No specific tumor antigens were demonstrated in the lymphomas used.

Paradise and Nungester (1966) reported a soluble antigen from a particulate fraction of mouse lymphosarcoma GC3HED. The antigenic substance appeared to be a protein-lipid-carbohydrate complex. All three components were necessary for maximum adsorption of rabbit antilymphosarcoma antiserum.

Strausser and Goldman (1965) evaluated eleven different tumors as to antigen specificity. Normal tissues were used to adsorb the anti-tumor antisera. Antigens specific for the tumors were demonstrated in all tissues.

Tumor specific antigens have been demonstrated by many investigators using various methods. In this study an attempt was made, using immunoelectrophoretic techniques, to demonstrate antigens unique to lymphomatous lymph node tissue.

MATERIALS AND METHODS

Source of specimens.

Normal lymph nodes (NLN) were obtained from 11 normal dogs owned by the Upjohn Co., Kalamazoo, Michigan. After removal, the lymph nodes were placed in a container with dry ice and stored at -70 C.

Normal canine serum was obtained from 10 normal dogs prior to surgery at the Michigan State University Veterinary Clinic.

Lymph nodes were obtained from 24 dogs with histologically diagnosed malignant lymphoma. These lymph nodes will be abbreviated (LLN). The lymph nodes were placed in containers with dry ice and stored at -70 C.

Serum was obtained from 7 dogs with histologically diagnosed malignant lymphoma. The lymph nodes and serum from dogs with lymphoma were obtained from the NIH project (PH 43-65-100) for leukemia transmission studies in the dog at Michigan State University.

Preparation of anti-normal canine serum antiserum (rabbit origin) (NCSAS).

Blood was obtained from the dogs by venipuncture, placed in tubes and allowed to clot. The tubes were

centrifuged, the serum removed, pooled, dispensed in 13 ml amounts and stored at -70 C until used.

Inocula were prepared as follows (Hirschfeld, 1960):

12.5 ml canine serum

40.0 ml distilled water

45.0 ml 10% $KAl(SO_4)_2 \cdot 12H_2O$

The mixture was adjusted to pH 6.5 with 5N NaOH, centrifuged and washed twice with a 0.85% saline solution. The sediment was made up to a volume of 50 ml with the saline solution.

The mixture was injected into 6 Dutch rabbits according to the following schedule (Hirshfeld, 1960):

Day 1: 4 ml intramuscularly into each buttock

Day 14: 4 ml intramuscularly into each buttock

Day 28: 4 ml intramuscularly into each buttock

Day 38: 1 ml whole serum intraperitoneally

Five days after the last injection all rabbits were bled by cardiac puncture. After the blood clotted, it was centrifuged and the serum removed. Each serum was tested by the ring precipitin test for cross-reactions with other sera. Two sera that cross-reacted were discarded. The other four sera were pooled, dispensed in 1 ml amounts and stored at -70 C.

Preparation of anti-lymphomatous canine serum antiserum (rabbit origin) (LCSAS).

Blood was obtained from 7 dogs with histologically

diagnosed malignant lymphoma by catheterization of the carotid artery following terminal surgery. After the blood clotted and was centrifuged, the serum was removed, pooled, dispensed in 13 ml amounts and stored at -70 C. Sera were prepared, tested and stored as described in the previous section.

Preparation of anti-lymphomatous lymph node antisera (LLNAS) and anti-normal lymph node antisera (NLNAS).

The lymph nodes were trimmed free of fat while frozen, minced and ground in a tissue grinder. To each milliliter of lymph node tissue, 0.02 ml of 10% $AlCl_3$ was added and the pH adjusted to 7.0 with 10% NaOH. A volume of the material containing 0.2 gm of lymph node tissue was injected subcutaneously into four areas on the back of each of 6 rabbits according to the schedule given for the preparation of NCSAS. The fourth inoculum did not contain $AlCl_3$ and was injected intraperitoneally.

Five days after the last injection the rabbits were bled by cardiac puncture. After the blood clotted and was centrifuged, the serum was removed and tested for cross-reactions as described previously. Two of the sera from rabbits inoculated with the lymphomatous lymph nodes cross-reacted and were discarded. The sera from the other 4 rabbits were pooled, dispensed in 1 ml amounts and stored at -70 C. No cross-reactions occurred with the sera from rabbits injected with normal lymph nodes. They were pooled, dispensed in 1 ml amounts and stored at -70 C.

Preparation of extract of lymph nodes for electrophoresis.

Lymph nodes were trimmed free of fat, minced and ground with a small amount of 0.85% saline in a tissue grinder. The ground lymph node material was centrifuged and the supernatant fluid was used for electrophoresis.

Immunoelectrophoresis.

The modified Spinco Durham cell[a] electrophoresis chamber was modified by reducing inside air space to lessen evaporation (Figure 1). Standard 1x3-inch glass microscope slides were coated with 2.5 ml of a 0.7% Agarose[b] solution in 0.0375 ionic strength veronal buffer of pH 8.5. The buffer was prepared by diluting 1 part of B-2 buffer[a] with 1 part of distilled water. After the Agarose solidified on the glass slides at 4 C, antigen wells and an antiserum slot were cut into the hardened Agarose with a device made for cutting reproducible patterns of the wells and antiserum troughs (Figure 2).

An extract of ground lymphomatous lymph node (LLN) was placed in one antigen well and an extract of normal lymph node (NLN) was placed in the other antigen well. The slides were placed across the end baffles of the chamber. Contact between the buffer and Agarose was accomplished by the use of filter paper wicks. The slides were electrophoresed for 60 minutes at 40 ma of constant current. After separation of the protein components of

[a]Beckman Instruments, Inc., Fullerton, Calif.

[b]Bausch and Lomb, Inc., Rochester, New York

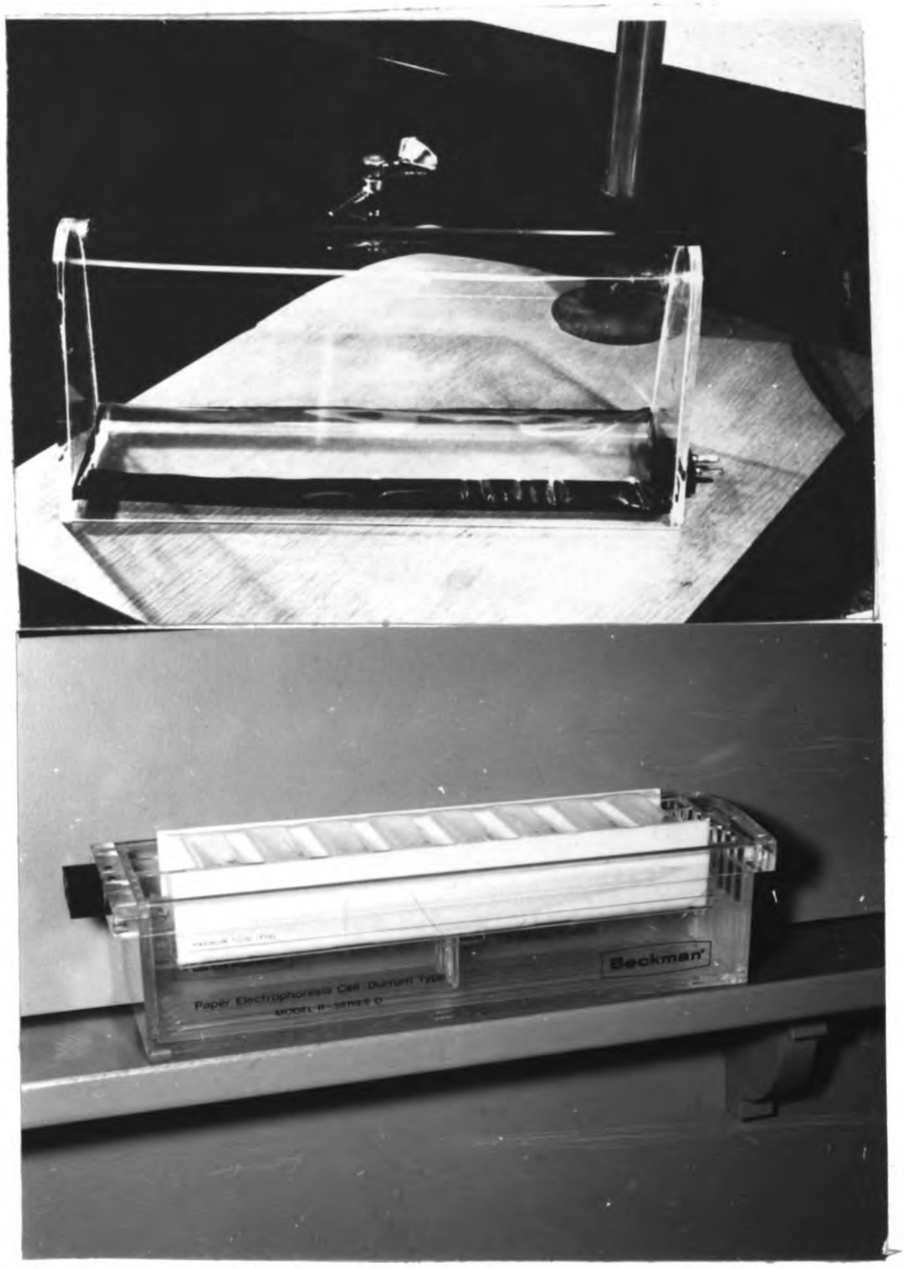

Figure 1. Electrophoresis chamber modified to accommodate 1x3-inch agar-coated glass slides. Top, cell cover with a plastic sheet taped inside to reduce evaporation by reducing the air space. Bottom, cell showing paper wicks and slides in place.

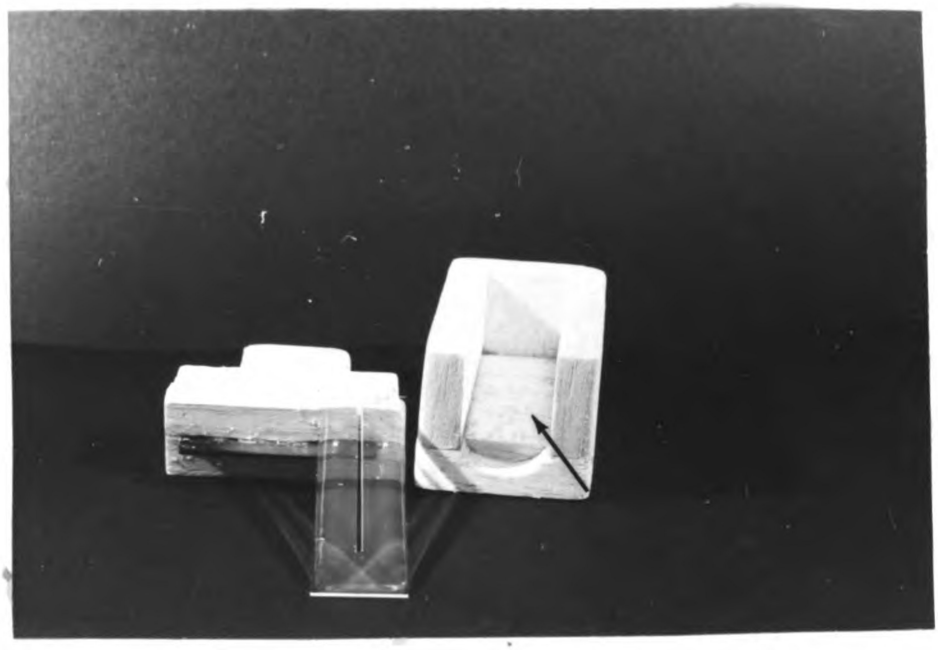

Figure 2. Agar cutter. An agar-coated slide (front, center) was placed in the chamber (arrow) and the plunger was pressed down to cut wells and trough for immunoelectrophoresis.

each of the lymph node extracts, the agar which up to now had been in the pre-cut antisera trough was removed and 0.1 ml of the antiserum being studied was added to the trough of each of the slides. The slides were placed in a moist, airtight container and incubated at 37 C for 18 hours for development of immunoprecipitates. After development of the arcs, the slides were photographed. Following photography, the slides were washed with several changes of an 0.85% saline solution. Washing was completed in approximately 24 hours. The slides were then washed in distilled water for 4 to 6 hours. After drying at 37 C, the slides were stained with a trichrome stain described by Crowle (1961) in order to permanently preserve the results previously photographed.

RESULTS

Lymphomatous canine serum antiserum (LCSAS).

The results using LCSAS were quite variable especially when individual normal lymph nodes were used instead of a pool of normal lymph nodes (Table 1). Immunoprecipitates occurred with four of five normal lymph node pools which did not occur with the lymphomatous lymph nodes. Using individual normal lymph node extracts, only two of seven lymph nodes developed arcs with the antiserum which were not present with the lymphomatous lymph node extracts.

Three of the 12 lymphomatous lymph node extracts formed immunoprecipitates with this antiserum that did not form with normal lymph nodes. These arcs were found in the intermediate (between gamma and albumin) region.

Representative immunoelectrophoretic patterns developed with LCSAS are depicted (Figure 3).

Normal canine serum antiserum (NCSAS).

The results using this antiserum were similar to those using LCSAS. Superior results were obtained using a pool of normal lymph nodes rather than using the individual lymph nodes.

TABLE 1. Results of immunoelectrophoretic studies of normal lymph nodes (NLN) and lymphomatous lymph nodes (LLN) developed with lymphomatous canine serum antiserum (LCSAS).

Dog No./ NLN* No.	No. immunoprecipitates with NLN	No. immunoprecipitates with LLN**	No. immunoprecipitates with NLN and not with LLN	Region of arc shown in previous column	No. immunoprecipitates with LLN and not with NLN	Region of arc shown in previous column
10020/5	4	4	0	-----	0	-----
10024/2	2	2	1	gamma	1	intermediate
10027/4	2	2	1	gamma	1	intermediate
10017/10	4	4	0	-----	0	-----
10018/10	2	2	0	-----	0	-----
10000/3	2	2	0	-----	0	-----
10/6	2	3	0	-----	1	intermediate
22/pool	5	2	1 2	gamma intermediate	0	-----
14/pool	5	3	1 1	gamma albumin	0	-----
16/pool	5	2	2 1	gamma albumin	0	-----
13/pool	4	4	0	-----	0	-----
32/pool	4	3	1	gamma	0	-----

* NLN - normal lymph node
** LLN - lymphomatous lymph node

Figure 3. Lymphomatous lymph node (LLN) and normal lymph node (NLN) extracts with immunoprecipitates developed against lymphomatous canine serum antiserum (LCSAS). In the upper half of each picture are arcs developed against NLN. In the lower half of each picture are arcs developed against LLN.

a) Identical immunoelectrophoretic patterns.

b) The arrows point to immunoprecipitates which occurred with NLN and not with LLN.

Numbers in parentheses indicate the lymphomatous lymph node number and normal lymph node number.

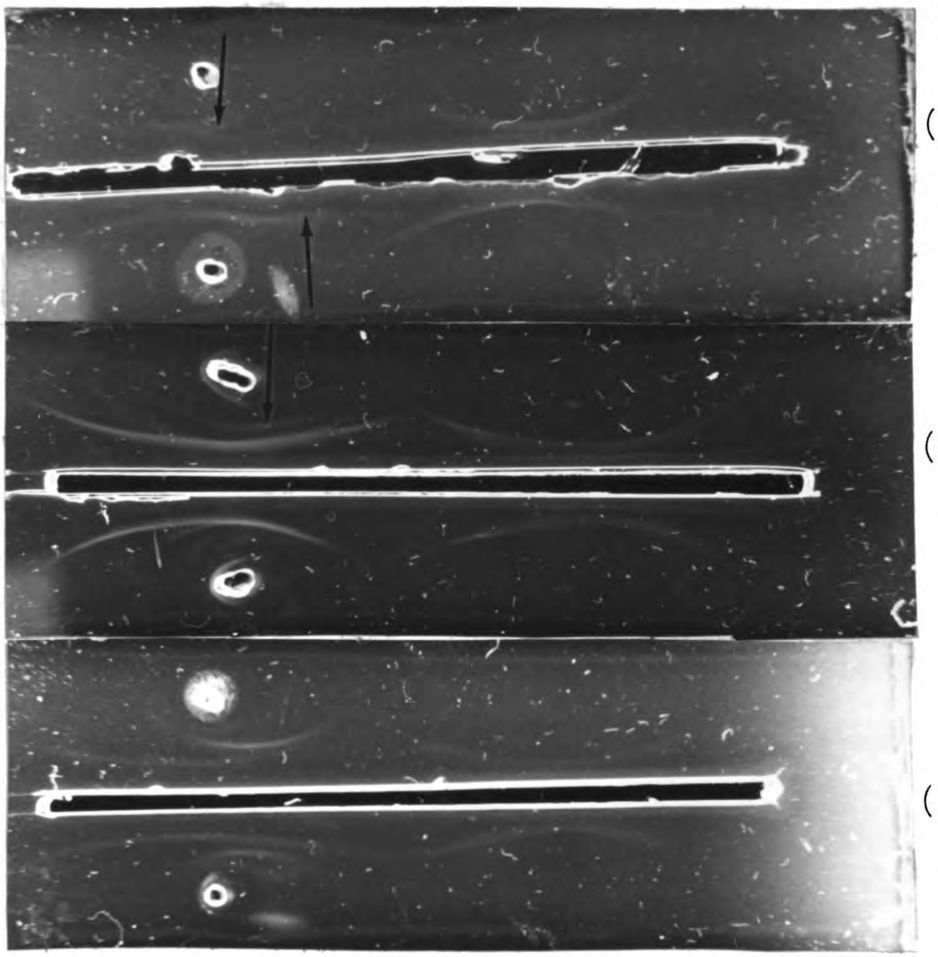

Figure 5.

Three of the 14 lymphomatous lymph node extracts developed arcs with the antiserum that were not present with normal lymph nodes. These arcs developed in the gamma and intermediate regions (Table 2). Representative immunoelectrophoretic patterns of NLN and LLN developed with NCSAS are depicted (Figure 4).

Lymphomatous lymph node antiserum (LLNAS).

The immunoprecipitates formed with LLNAS are shown in Table 3. Fewer arcs developed with this antiserum than with LCSAS or NCSAS. Also, fewer NLN and LLN had arcs that were unique to each node. Arcs that were present with NLN and not with LLN and arcs present with LLN and not with NLN were all in the gamma region.

Photographs of representative immunoelectrophoretic patterns developed with LLNAS are shown in Figure 5.

Normal lymph node antiserum (NLNAS).

Five of 12 normal lymph node extracts developed immunoprecipitates with NLNAS (Table 4).

Two of 12 lymphomatous lymph nodes developed arcs with the antiserum that did not occur with the normal lymph node. These arcs were present in the gamma region.

Photographs of representative immunoelectrophoretic patterns developed with NLNAS are shown in Figure 6.

TABLE 4. Results of immunoelectrophoretic studies of normal lymph nodes (NLN) and lymphomatous lymph nodes (LLN) developed with normal lymph node antiserum (NLNAS).

Dog No./ NLN* No.	No. immunoprecipitates with NLN	No. immunoprecipitates with LLN**	No. immunoprecipitates with NLN and not with LLN	Region of arc shown in previous column	No. immunoprecipitates with LLN and not with NLN	Region of arc shown in previous column
10022/1	3	2	1	gamma	0	-----
11/6	3	3	0	-----	0	-----
18/10	3	2	1	gamma	0	-----
27/4	3	2	1	gamma	0	-----
37/3	3	2	1	gamma	0	-----
20/1	4	4	0	-----	0	-----
6/2	3	4	0	-----	1	gamma
25/4	4	3	1	gamma	0	-----
17/5	3	3	0	-----	0	-----
32/6	4	4	0	-----	0	-----
19/2	2	2	0	-----	0	-----
16/7	2	4	0	-----	1 1	gamma albumin

* NLN - normal lymph node
** LLN - lymphomatous lymph node

Figure 6. Lymphomatous lymph node (LLN) and normal lymph node (NLN) extracts showing immunoprecipitates developed with lymphomatous lymph node antiserum (LLNAS). In the upper half of each picture are arcs developed against NLN. In the lower half are arcs developed against LLN.

a) The arrow points to an immunoprecipitate which occurred with NLN and not with LLN.

b) Identical immunoelectrophoretic patterns.

c) The arrow points to an immunoprecipitate which occurred with LLN and not with NLN.

d) The arrow points to an immunoprecipitate which occurred with NLN and not with LLN.

Numbers in parentheses indicate the lymphomatous lymph node number and normal lymph node number.

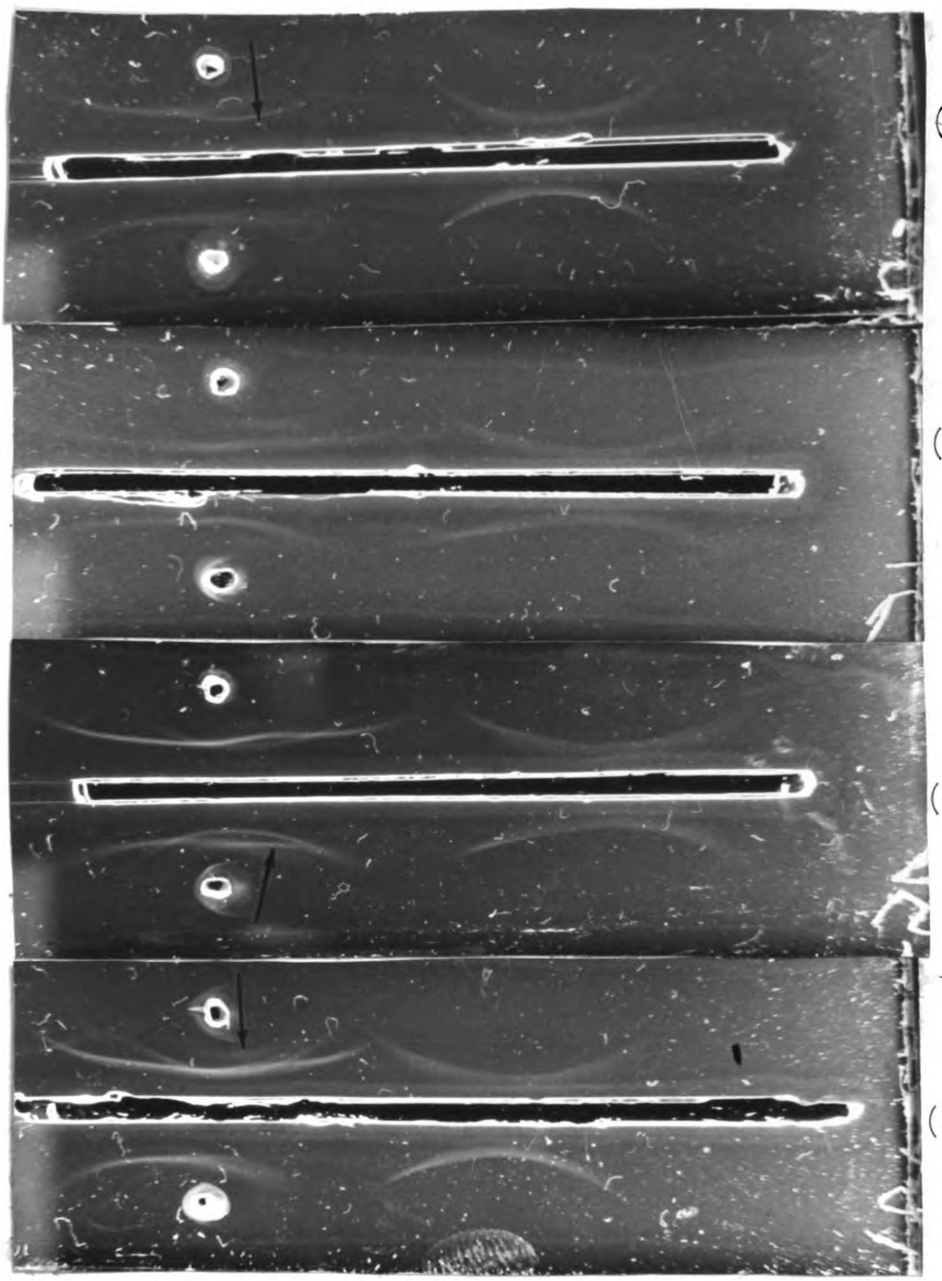

Figure 6.

DISCUSSION

A reliable interpretation of antigen-antibody reactions requires an awareness of the limitations of a given system. The Ouchterlony gel diffusion method is not the most sensitive serologic test. For example, complement fixation and passive hemagglutination methods are more sensitive than precipitation techniques (Marrack, 1963). However, the gel diffusion tests have the distinct advantage of detecting and delineating multiple antigen-antibody systems and determining the relationship of these systems to each other. Passive hemagglutination and complement fixation tests do not have these advantages and they are technically more cumbersome and require elaborate control systems.

The antigens used in this study were complex mixtures of multiple antigens. Undoubtedly, the optimal conditions for any given antigen-antibody system could vary and the optimum could not be obtained for all systems simultaneously. Gel double diffusion methods, with the antigen and antibody diffusing toward each other, theoretically allow each system to seek its own equivalence zone. Thus the possibilities for detecting various antigen-antibody systems appeared to be greater using a gel diffusion method rather than another serological procedure.

There are numerous possible combinations of results for any given immunoprecipitate. The remainder of this discussion will deal with possible results demonstrating the "loss" of an antigen and demonstrating a "new" antigen. These theoretical results will then be applied to the actual results obtained during this study.

Possible results demonstrating "loss" of an antigen.

If a given immunoprecipitate developed with an extract of NLN and not with an extract of LLN using NLNAS, this would suggest a "loss" of a normal antigen in the LLN. However, using LLNAS a negative result with an extract of LLN and a positive result with an extract of NLN would imply that the apparent loss of an antigen is probably due to the normal antigen being present in too low concentration in the LLN to react in the system used.

Possible results demonstrating a "new" antigen.

If a given immunoprecipitate developed with an extract of LLN and not with an extract of NLN using LLNAS, this would probably indicate a "new" antigen in the LLN. The possibility exists, however, that the antigen may be present in the NLN, but at too low a concentration to react. This would be unlikely because one would expect that the NLN would have antigen in concentration adequate to react in the precipitin system.

An immunoprecipitate with an extract of NLN and none with an extract of LLN using NLNAS would not exclude the possible presence of a "new" antigen. This result was

postulated for the loss of an antigen, but the loss of a normal antigen could indicate the presence of a new antigen whose antibody was not present in the NLNAS. Again the possibility exists of the antigen being present in too low concentration to react.

Possible results indicating identical or cross-reacting antigens.

If a given immunoprecipitate developed with extracts of both NLN and LLN using either LLNAS or NLNAS the obvious answer would be that the lymph nodes have identical antigens. However, one of the antigens may be different, but similar enough to react with the antiserum used. This type of reaction would then indicate either identical or similar antigens on both NLN and LLN.

The above possibilities mentioned for LLNAS and NLNAS could also be applied to LCSAS and NCSAS. Using LCSAS and developing an immunoprecipitate with LLN and not with NLN would imply a circulating antigen in dogs with malignant lymphoma, or an antigen in the NLN with too low concentration to react with the LCSAS. An immunoprecipitate present with NLN and not with LLN using NCSAS indicates a loss of an antigen in the LLN. Again, this may be due to antigen concentration.

As previously stated, the results using individual lymph nodes were more variable than when pools of normal lymph nodes were used. Therefore, pooled tissues rather than individual tissues should be used for control purposes.

Three immunoprecipitates were present with LLN extracts and not with NLN extracts (Table 4). As previously discussed, this indicates the possibility of a unique circulating antigen in dogs with malignant lymphoma. However, with the small sample used (12 lymph nodes) it would be presumptuous to make a clear-cut statement to this effect.

Eight of the 14 lymph nodes developed immunoprecipitates with NLN extracts and not with LLN extracts using LCSAS (Table 2). This is a strong indication of the loss of an antigen and in some instances the loss of antigens in the LLN.

Two immunoprecipitates were present with LLN extracts and not with NLN extracts (Table 3). Since LLNAS was used this demonstrates the presence of a "new" antigen in the LLN. This table also demonstrates the results of too low antigen concentration since two of the NLN developed arcs with the antiserum that were not present with LLN.

Five immunoprecipitates were present in NLN extracts that were not present with LLN extracts (Table 4). This indicates the probable loss of a normal antigen in the LLN Two LLN developed immunoprecipitates with LLN and not with NLN. Since NLNAS was used, the results were, in all probability, due to too low antigen concentration in the NLN.

Adsorption of the various antisera used in this study with appropriate tissue antigens might have improved the results obtained. Concentration of the various antigens by chemical means might have resolved the antigen

concentration difficulty that was encountered. The indication that LLN have altered antigenic components encourages further study in this area.

SUMMARY

The object of this study was to determine if lymph node cells from dogs with malignant lymphoma had an antigen or antigens that were not present on normal lymph node cells. The results indicated a change in the antigenic structure of canine lymphomatous lymph node cells. This change was evidenced by the absence of "normal" antigens or by the presence of a unique antigen or antigens on the lymphomatous lymph node cells.

The antigenic changes were detected using normal canine serum antiserum (rabbit origin), lymphomatous canine serum antiserum (rabbit origin), lymphomatous canine lymph node antiserum (rabbit origin) and normal canine lymph node antiserum (rabbit origin) to develop immunoprecipitates with normal canine lymph nodes and lymphomatous canine lymph nodes using immunoelectrophoretic techniques.

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