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Alkaline and Acid Phosphatase in Murine Leukemia

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Alterations for acid and alkaline phosphatase levels and their pattern of splenic and lymph node activity in normal and virus-induced lymphoblastic leukemia were studied. Enzyme levels were examined by using both cytochemical and biochemical procedures. The GC leukemia virus, a ribonucleic acid murine virus antigenically related to the Rauscher-Moloney viruses, was used to stimulate acid and alkaline phosphatase by producing lymphomaceous disease in Ha/ICR mice. With the Burstone and Gomori cytochemical procedures, both enzymes were found in higher than normal levels in lymphomaceous spleen and lymph nodes. Confirmation of the cytochemical studies was obtained by enzyme assay of cell-free homogenates in each case with the exception of spleen acid phosphatase. The discrepancy between the cytochemical tests which showed significant elevation of spleen acid phosphatase and the enzyme assays which failed to reveal such elevation could be due to a labile acid phosphatase isozyme which is lost on cellular disruption during homogenate preparation. A significant spleen alkaline phosphatase specific activity elevation above normal was found with a 50% incidence only when leukemic spleen wet weight increased nearly threefold its normal value. This result suggests that alkaline phosphatase elevation is a secondary event occuring after the onset of disease and is not a fundamental metabolic alteration concerned with the onset of murine lymphoblastic leukemia.

Several studies have demonstrated elevation of alkaline phosphatase in leukemic mouse reticuloendothelial organs (7, 11, 14-16, 19-21). Also, higher amounts of acid phosphatase have been detected in peripheral blood cells following Rauscher leukemia virus infection (12). Such studies on enzymes and their alterations in malignant cells should lead to a better understanding of neoplasia, a prerequisite for successful therapy. Accordingly, in the present investigation, alterations of both acid and alkaline phosphatase levels and their pattern of activity in spleen and lymph node cells of mice with a viral-induced lymphoblastic leukemia have been examined. Both cytochemical and biochemical procedures were employed. The lymphoma virus used, designated GC (10), is of the ribonucleic acid (RNA) type (2) and antigenically related to the Friend-Moloney-Rauscher group (3).

MATERIALS AND METHODS

Animals. Two to 4-day-old random-bred Ha/ICR Swiss mice were inoculated intraperitoneally with GC virus as previously described (10). Animals were sacrificed by cervical compression, and the organs were quickly excised, weighed, placed on ice, and used within 1 hr for enzyme studies.

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Cytochemical procedures. The calcium cobalt technique of Gomori (9), its modification as recommended by Pearse (17), and Burstone's azo-dye staining (4) were used to study alkaline phosphatase. Prior to treatment by Gomori's method, impression smears were frozen in powdered dry ice for 5 min. After fixation in cold (4 C) absolute acetone for 24 hr, they were incubated from 30 min to 16 hr at 37 C. Preparations processed by Pearse's modification were fixed in 90% ethanol for 24 hr at ambient temperature and then incubated from 10 to 60 min at 37 C. The mounting medium used was Harleco synthetic resin. Naphthol AS-MX phosphate served as substrate, and Red Violet-LB salt was the azo-dye in Burstone's procedure. Unfixed impression smears were incubated from 30 min to 16 hr at 37 C. The mounting medium was polyvinylpyrrolidone (PVP).

Gomori's lead nitrate method, as given by Pearse (17), and Burstone's azo-dye technique (4) were used to study acid phosphatase. Smears processed by the method of Gomori were placed at −70 C for 1 hr (Revco deep freeze) and then incubated from 30 min to 16 hr at 37 C. By Burstone's technique, unfixed impression smears were incubated at 37 C from 30 min to 6 hr in a medium containing naphthol AS-BI phosphate and Red Violet-LB salt. Preparations incubated by the method of Gomori were mounted in glycerine jelly and those by Burstone were mounted in PVP.

Homogenates. Lymph nodes or spleen in ice-cold 0.15 M KCl [1 g (wet weight) tissue/5 ml of salt solu-

tion] were homogenized with an all-glass Dounce device. Homogenization was started with 10 up-and-down strokes with the loosely fitting pestle and completed similarly with 10 strokes of the tightly fitting pestle. Cell breakage was essentially complete as observed microscopically. Alternatively, homogenization was achieved by agitation for 3 min in a Waring blendor followed by sonic treatment for 5 min. Homogenates prepared by either method gave comparable phosphatase assay values.

Assays. For alkaline phosphatase activity, reaction mixtures contained 10 μmoles of *p*-nitrophenylphosphate (PNPP), 200 μmoles of $MgCl_2$, 200 μmoles of tris(hydroxymethyl)amino - methane - hydrochloride buffer at *p*H 9.1 and enzyme in a final volume of 1 ml. For acid phosphatase, reaction mixtures contained 10 μmoles of PNPP, 50 μmoles of sodium acetate buffer (*p*H 5.0), 0.1% Triton X-100 to measure total activity (1), and enzyme in a final volume of 1 ml. Reaction mixtures were modified by incorporation of 10 μmoles of sodium β-glycerolphosphate or 3 μmoles of naphtholphosphate to measure phosphatase activity with these as substrates. Incubations were from 30 min to 1 hr at 37 C. Liberated *p*-nitrophenol was estimated at 410 nm in 0.15 N NaOH. With β-glycerolphosphate and naphtholphosphate, the reactions were stopped with 1 ml of 20% trichloroacetic acid. Following filtration, inorganic phosphate was estimated by the procedure of Fiske and SubbaRow (8). Activities are expressed as nanomoles of product liberated per minute per milligram of protein. Protein was estimated (13) with crystalline bovine serum albumin as standard.

RESULTS

Passage of GC virus in newborn Ha/ICR mice produced a leukemia incidence of about 90% with a latency period ranging from 40 to 60 days. The resulting lymphoma, as observed in reticuloendothelial tissue, is an undifferentiated stem cell tumor containing large numbers of lymphoblasts (Fig. 1). This neoplastic proliferation of lymphoblasts was accompanied by organ enlargement ranging up to 14 times the normal spleen weight and nearly 40 times the normal lymph node weight.

Cytochemical determination of alkaline phosphatase by both the Burstone and Gomori procedures showed this enzyme to be present in greater than normal amounts in lymphomaceous spleen and lymph nodes. With short incubation times (30 min to 1 hr, Burstone procedure), enzyme was localized mainly in discrete granules found at the periphery of leukemic cells (Fig. 2). Longer incubation periods (to 6 hr) resulted in the additional appearance of positively staining granules scattered throughout the cytoplasm in both normal and leukemic cells, but total activity was consistently greater in lymphomaceous spleen and lymph nodes. With spleen smears prepared during the latency period (same animals and time intervals as shown in Table 2), a difference in staining reaction between normal and inoculated animals was not detected (Burstone procedure, incubations for 30 min).

Burstone's technique for acid phosphatase reveals activity to be localized mainly in discrete round granules scattered throughout the cytoplasm of both normal and leukemic cells. These granules probably reflect a lysosomal location for acid phosphatase (5). Although the number of granules did not vary significantly between normal and leukemic cells, staining intensity was greater than normal (20 smears studied) in 20 out of 23 (87%) lymphomaceous spleen smears examined. Gomori's procedure (Fig. 3) revealed greater than normal amounts of acid phosphatase in all leukemic lymph nodes and spleen smears studied (four normal and four lymphomaceous preparations of each organ). As with alkaline phosphatase, spleen smears prepared during the latency period (same animals and time intervals as shown in Table 2) failed to show alteration in either the amount or distribution of acid phosphatase between inoculated and normal mice.

Cell-free homogenates of normal and lymphomaceous organs were compared for alkaline phosphatase activity. Substrates employed included PNPP as well as the same phosphate esters used for cytochemical analysis, β-glycerolphosphate and naphtholphosphate. These results (Table 1) show the specific activity for leukemic spleen and lymph node alkaline phosphatase to be substantially elevated above normal with all three substrates. When spleen homogenates prepared from normal and inoculated animals during the latency period were tested (PNPP as substrate), some variation of enzyme specific activity was noted (Table 2); however, only in leukemic animals when spleen wet weight was frankly increased above normal did the level of alkaline phosphatase rise substantially.

A total of 37 spleens from leukemic animals (average wet weight, 915 mg; range, 280 to 1,750 mg) and 13 from normal animals (average wet weight, 200 mg; range, 120 to 340 mg) were tested individually for alkaline phosphatase specific activity. These results (Fig. 4) show alkaline phosphatase levels are generally elevated in spleen lymphoma (27/37 = 73%) but with considerable variation in specific activity. This variation was not the result of animal sex or age or spleen protein levels. Spleen protein content differed little between lymphomaceous [average, 141 mg of protein per gram (wet weight) of spleen; range, 123 to 164] and normal [average, 153 mg of protein per gram (wet weight) of spleen; range 133 to 185]. With each spleen tested, excellent correlation was obtained between cytochemical results (Burstone method,

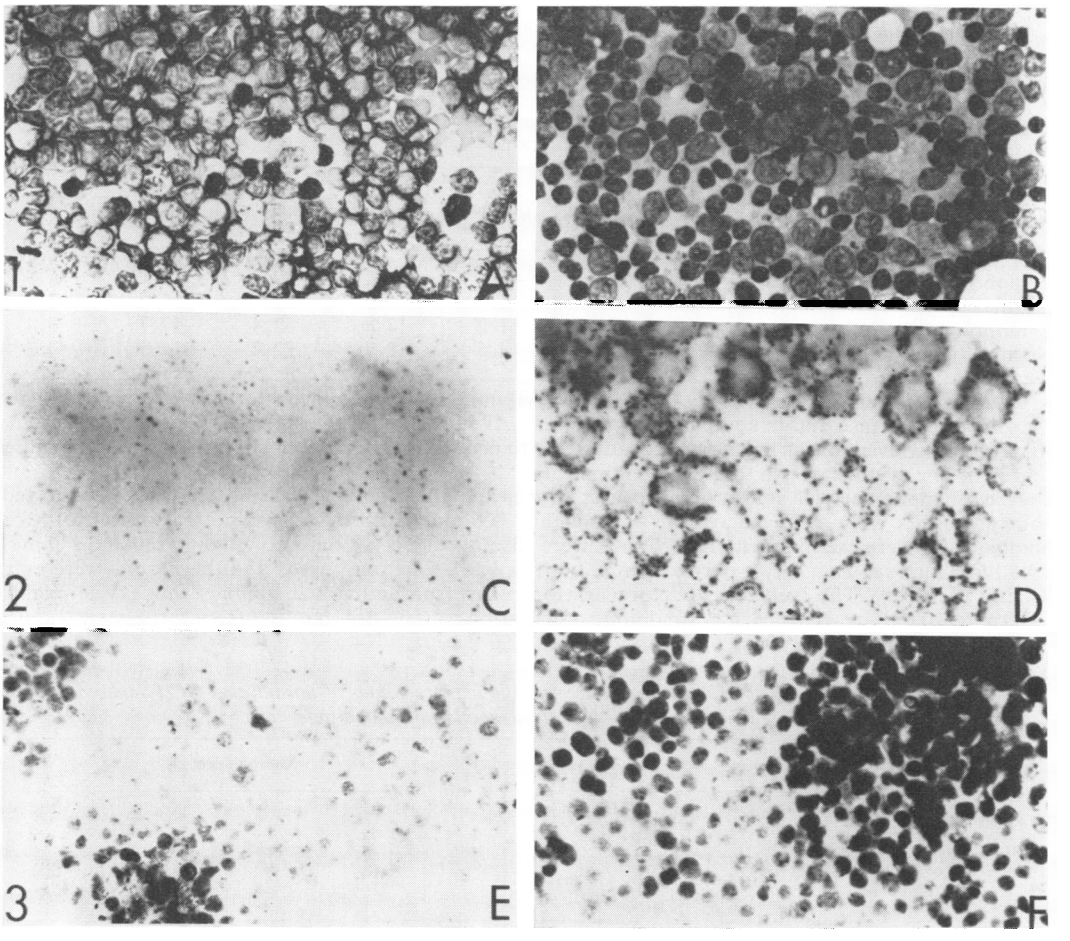

FIG. 1. *Giemsa-stained impression smears from 60-day-old Ha/ICR mice. × 160. (A) Normal spleen; (B) spleen from a leukemic animal which had been inoculated at 4 days of age with GC lymphoma virus.*

FIG. 2. *Alkaline phosphatase in normal (C) and lymphomaceous (D) spleen of Ha/ICR mice. × 400. Burstone's procedure, 30 min of incubation at 37 C. Both animals were 65 days of age. The leukemic mouse had been inoculated with GC lymphoma virus at 4 days of age.*

FIG. 3. *Acid phosphatase of normal (E) and lymphomaceous spleen (F) of Ha/ICR mice. × 160. Gomori's method, 5 hr of incubation at 37 C. Spleens from the same animals described in Fig. 2 were employed.*

30-min incubation time) and alkaline phosphatase specific activity.

Although no direct correlation between individual spleen wet weight and alkaline phosphatase specific activity was obtained, it was observed that with lymphomaceous spleens weighing over 800 mg, 21/25 (84%) showed an alkaline phosphatase specific activity elevation of twofold or more above the average control value. With spleens weighing from 400 to 600 mg, 6/12 (50%) had a similar elevation, but, with those weighing from 200 to 400 mg, only 2/8 (25%) had a twofold or greater elevation of alkaline phosphatase specific activity.

In the case of acid phosphatase, a specific activity increase in lymphomaceous spleen homogenates was never observed. A total of 11 normal spleen homogenates showed an average acid phosphatase specific activity (PNPP) of 36.6 nanomoles per min per mg of protein (range, 25.6 to 44.5), whereas a total of 18 lymphomaceous spleen homogenates exhibited an average specific activity of 30.0 (range, 19.4 to 43.1). A significant elevation was, however, detected for lymphomaceous lymph node acid phosphatase specific activity with PNPP as well as with β-glycerolphosphate and naphtholphosphate (Table 1). As with alkaline phosphatase, there was no activity alteration for acid phosphatase (as determined either cytochemically or by en-

TABLE 1. *Specific activity for alkaline and acid phosphatase of lymphomaceous and normal mouse spleen, and lymph node homogenates by using three phosphate esters*

Organ[b]	Specific activity[a]					
	Alkaline phosphatase			Acid phosphatase		
	p-Nitrophenyl-phosphate	β-Glycerol phosphate	Naphthol-phosphate	*p*-Nitrophenyl-phosphate	β-Glycerol-phosphate	Naphthol-phosphate
Lymphomaceous spleen	45.6	46.8	9.4	30.2	16.1	6.5
Normal spleen	6.6	6.4	1.6	35.4	15.0	7.5
Lymphomaceous lymph node	88.1	61.2	17.8	32.4	17.7	6.4
Normal lymph node	10.8	14.5	2.7	22.7	14.5	3.2

[a] Substrates were assayed in tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 9.1) for alkaline phosphatase and in sodium acetate buffer (*p*H 5.0) for acid phosphatase. One unit of activity signifies the conversion of 1 nanomole of substrate to product per min per mg of protein at 37 C. Values represent the average of two to five determinations.

[b] Two lymphomaceous spleen homogenates with nearly the same level of phosphatases were tested, and the results were averaged (spleen wet weights, 800 and 1,050 mg). Two normal spleen cell-free homogenates were examined, and the results were also averaged (spleen wet weights, 180 and 140 mg). Two lymphomaceous and two normal lymph node preparations were used, and the results were averaged in each case. The leukemic node preparations were from individual animals (node wet weights, 1,500 and 820 mg), whereas each normal node preparation was pooled material from three mice (pooled node wet weights, 100 and 90 mg).

TABLE 2. *Spleen wet weight and phosphatase specific activity values of normal and GC lymphoma virus-inoculated mice as a function of animal age*

Infected mice[a]					Normal mice				
Age (days)	No. of spleens tested	Spleen wet weight (mg)	Phosphatase specific activity[b]		Age (days)	No. of spleens tested	Spleen wet weight (mg)	Phosphatase specific activity	
			Alkaline (units/mg)	Acid (units/mg)				Alkaline (units/mg)	Acid (units/mg)
9	1	30[c] (0)[d]	9.6 (0)	46.6 (0)	9	1	25 (0)	12.0 (0)	46.5 (0)
15	3	52 (35–70)	9.2 (7.2–11.9)	37.6 (31.8–45.5)	15	3	45 (25–80)	12.6 (7.2–15.5)	40.0 (31.1–46.6)
18	2	70 (50–90)	7.0 (4.8–9.3)	34.1 (29.9–38.2)	18	2	50 (0)	7.2 (0)	43.0 (0)
21	2	108 (100–115)	5.1 (0)	30.1 (29.0–31.6)	21	2	90 (0)	12.3 (7.9–16.7)	24.5 (21.5–27.4)
25	3	100 (50–150)	9.2 (7.4–10.7)	32.5 (22.9–45.5)	25	3	163 (90–180)	11.9 (9.1–16.8)	36.3 (33.5–37.1)
29	2	120 (100–140	7.9 (7.6–8.1)	38.1 (34.0–42.1)	29	3	170 (120–260)	10.0 (8.8–12.0)	42.8 (41.0–44.5)
36	3	180 (160–190)	6.7 (5.3–8.4)	29.6 (25.6–35.8)	36	3	280 (210–290)	7.3 (5.7–10.0)	54.5 (38.2–51.2)
40	2	265 (210–320)	9.1 (7.9–10.2	32.1 (28.6–35.5)	40	3	233 (210–260)	6.1 (5.3–6.7)	47.8 (34.3–51.9)
48	4	712 (400–1,390)	14.5 (8.1–28.8)	32.0 (26.4–41.5)	48	1	200 (0)	11.9 (0)	40.6 (0)
60	4	1,320 (900–1,800)	30.6 (12.9–62.0)	30.7 (23.9–35.8)	60	4	202 (130–300)	12.3 (8.7–14.6)	39.5 (26.4–44.5)
74	4	890 (300–1,500)	25.2 (4.5–41.1)		74	2	190 (180–200)	9.5 (9.1–10.0)	32.9 (31.2–34.6)

[a] Infected Ha/ICR mice were inoculated at 2 days of age with GC lymphoma virus. A spleen impression smear of each infected animal tested was also examined for leukemia after Giemsa staining. From 9 to 40 days of age, no leukemia was evident. The 48-, 60-, and 74-day-old animals each had some degree of lymphoblastic leukemia.

[b] Cell-free homogenates were assayed in tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 9.1) for alkaline phosphatase and in sodium acetate buffer (*p*H 5.0) for acid phosphatase. One unit of activity signifies the release of 1 nanomole of *p*-nitrophenol per min per mg of protein at 37 C from *p*-nitrophenylphosphate.

[c] Average value.

[d] Numbers in parentheses equal range.

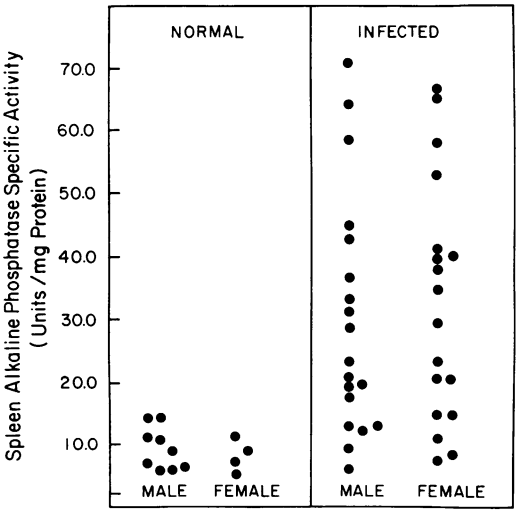

FIG. 4. *Alkaline phosphatase specific activity values for normal and lymphomaceous spleen as a function of animal sex.*

zyme assay) between spleens from normal and inoculated animals sacrificed during the latency period (Table 2). Because of the discrepancy between the cytochemical observations showing higher than normal levels of leukemic spleen acid phosphatase and the enzyme assay results showing no such increase, further studies were carried out. One explanation for this anomaly would be that leukemic spleen contains a labile acid phosphatase isozyme which is lost upon cellular disruption during homogenate preparation. Attempts to demonstrate the presence of such a fraction were unsuccessful; e.g., a difference in acid phosphatase specific activity between lymphomaceous and normal spleen homogenates was not detected following (i) preparation of homogenates in acidic buffers (0.05 M sodium citrate or 0.05 M sodium acetate); (ii) overnight dialysis against either of these buffers; (iii) assay as a function of time (10 min to 7 days) following homogenate preparation; (iv) incorporation into the reaction mixtures of ethylenediaminetetraacetic acid (1 to 10 μmoles), 2-mercaptoethanol (2 to 20 μmoles), or from 0.2 to 2 μmoles of manganese ions.

DISCUSSION

GC lymphoma virus produces a lymphoblastic leukemia similar to that caused by the Moloney, Gross, or Rich viruses (18), as judged by a prolonged latency period, reticuloendothelial organ enlargement after the onset of disease, and appearance of large numbers of lymphoblasts in the spleen and lymph node tumors.

During the latency period, significant differences were not detected between normal and inoculated mice for spleen wet weight, protein levels, or for acid and alkaline phosphatase (as determined either cytochemically or by enzyme assay of cell-free homogenates). Following the onset of leukemia, however, when spleen and lymph nodes were significantly enlarged above normal, phosphatase levels also changed. With alkaline phosphatase, cytochemical observations demonstrated significantly higher than normal amounts of enzyme in lymphomaceous spleen and lymph nodes of Ha/ICR mice. Short incubation times with the Burstone procedure revealed enzyme to be localized mainly in granules located at the periphery of leukemic cells. A peripheral location for alkaline phosphatase in leukemic cells has been previously reported (6, 19). In this study, longer incubation periods resulted in the additional appearance of positively staining granules scattered throughout the cytoplasm which were always more evident in lymphomaceous material. Cytochemical observations demonstrating elevation of alkaline phosphatase have been reported for thymus of both AKR and C57BL mice infected with other murine leukemia viruses (11, 15, 20, 21). In this study, enzyme assay procedures with PNPP as well as the same substrates employed for cytochemical analysis (β-glycerolphosphate and naphtholphosphate) confirm these cytochemical studies by demonstrating a higher than normal specific activity for lymphomaceous spleen and lymph node alkaline phosphatase. Although such increases were variable when lymphomaceous spleens were individually tested, the average elevations reported here (about fourfold for lymphomaceous spleen and eightfold for lymph nodes) are similar to those reported by Neumann et al. (16), who tested AKR mice with a spontaneous lymphoid leukemia and C57BL mice with a viral-induced lymphoid leukemia. Our results show that GC lymphoma virus in Ha/ICR mice behaves much as other murine leukemia viruses in causing significant elevation of alkaline phosphatase specific activity following the onset of murine leukemia.

The fact that a lymphomaceous spleen alkaline phosphatase elevation of at least twofold or greater above normal was observed with a 50% or greater incidence only when spleen wet weight was nearly threefold above normal is of interest. These data suggest that alkaline phosphatase elevation is not a fundamental metabolic alteration concerned with the onset of leukemia, but rather would represent a secondary change in spleen lymphoma occurring after the onset of neoplasia. This is in agreement with observations reported by other investigators (19) who were able to show by cytochemical methods that thymic alkaline phosphatase in Ha/ICR mice

(inoculated with the Rich lymphoma virus) was elevated only after the lymphoma had proliferated to the extent that this organ was at least threefold its normal weight.

It has been previously shown with both C57BL (14) and Ha/ICR (D. A. Wilson, H. Lo, and E. R. Brown, Bacteriol. Proc., p. 188, 1969) mice that only a single electrophoretic species (polyacrylamide gel) with the same mobility is found in both normal and lymphomaceous spleen. On this basis, it has been postulated that alkaline phosphatase elevation in murine leukemia is the result of derepression of a cell-coded enzyme during the process of neoplastic transformation (14). The data reported here, which show alkaline phosphatase specific activity elevation in lymphomaceous spleen is not the result of animal age or sex or due to differences between normal and leukemic spleen protein levels, support this view.

To our knowledge there has been only a single report on the elevation of acid phosphatase in viral-induced murine leukemia (12). In this instance, cytochemical studies demonstrated elevated acid phosphatase in erythroblastic murine leukemia following Friend virus inoculation. The cytochemical observations reported here by using two different methods (Gomori and Burstone) demonstrate consistent elevation of acid phosphatase in both spleen and lymph nodes following the onset of GC virus-induced lymphoblastic leukemia. Confirmation of these cytochemical observations by enzyme assays was obtained with lymph node homogenates by using PNPP as well as the same substrates employed for cytochemical analysis. With spleen homogenates, however, biochemical tests failed to demonstrate the acid phosphatase elevation detected cytochemically. This could be due to the presence of a labile acid phosphatase isozyme which is lost upon preparation of lymphomaceous spleen homogenates.

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