[CANCER RESEARCH 31, 1702–1705, November 1971]

Chronological Appearance of Alkaline Phosphatase Activity in Virus-induced Thymic Lymphomas of C57BL/6 Mice[1]

K. J. Wilson,[2] H. Neumann, and N. Haran-Ghera

Department of Biophysics (K. J. W., H. N.), and Department of Experimental Biology (N. H-G.), The Weizmann Institute of Science, Rehovot, Israel

SUMMARY

Mice of the C57BL/6 strain were inoculated with two different radiation leukemia viruses, and chronological changes in the alkaline phosphatase levels of the thymus, the spleen, the mesenteric lymph nodes, the liver, and the kidney were measured. The two radiation leukemia viruses used, one of which produces a lymphoma incidence of 80 to 100% after inoculation followed by X-irradiation and the other of which induces lymphatic leukemia in 80 to 100% of the inoculated mice without the need for further exposure to irradiation, were injected directly into one thymus lobe of adult mice, and at predetermined time intervals the alkaline phosphatase levels were measured. Elevation of the alkaline phosphatase activity occurred in the "target" tissue, the thymus, simultaneously or, more likely, prior to the development of the disease. The elevation of the alkaline phosphatase levels in the other tissues studied occurred only after dissemination had started.

INTRODUCTION

Histochemical studies (2, 4, 8), as well as biochemical characterizations (3, 5), have been carried out demonstrating the increase in APase[3] activity in the thymus of mice developing lymphatic leukemia. These observations were established in spontaneous leukemias in the AKR strain of mice (4, 5, 8), in radiation-, virus-, and chemical-induced leukemias in the C57BL strain of mice (2, 3, 5, 8) and in ICR/Ha Swiss mice inoculated with the Rich virus (7). An important question arising from these observations is whether the increase in APase activity in the thymus occurs at the latter stage of leukemic development (neoplastic stage) or if the APase activity appears before the disease can be histologically detected. A second question is whether the increase in APase activity occurs simultaneously with the development of the disease, exclusively in the thymus (the "target" tissue where the disease first develops), or whether the APase activities of other tissues such as the mesenteric nodes, the spleen, the liver, the kidney, and the lymph nodes also increase with the development of the disease prior to dissemination.

We felt that it was necessary to try to answer the questions presented above before attempting to understand the role of the increased APase activity in the thymus in relation to the development of the tumor itself. On the basis of these arguments, this paper reports the results of a time study concerning the levels of APase activity in different tissues of normal and leukemic mice (lymphatic leukemia being induced in these mice by the radiation leukemia virus) at various stages of development.

MATERIALS AND METHODS

Animals. Male mice of the C57BL/6 strain, from our inbred colony, were used when 6 to 8 weeks old. They were fed Purina laboratory chow and water *ad libitum.*

The Radiation Leukemia Virus. The preparation and inoculation of the viruses were carried out as previously described (6). Two different virus preparations were used in this study: (*a*) a virus preparation marked as "Passage 127." This virus, when injected directly into the thymus of adult mice, induces lymphomas in 15 to 25% of the inoculated mice. The incidence can be increased to 80 to 100% by further exposing the mice, within a few days after virus inoculation, to 400R of whole-body irradiation; (*b*) the other virus preparation used, marked as "Passage 136," when injected directly into an adult thymus induces lymphatic leukemia in 80 to 100% of the inoculated mice (without the need for further exposure to irradiation).

Lymphatic Leukemia Induction. The radiation leukemia virus Passage 127 (0.02 ml of 20% concentration in PBS) was injected directly into one thymus lobe of adult mice, and 2 days thereafter the mice were exposed to 400 R of whole-body irradiation.

The Viral Passage 136 was injected directly into one thymus lobe of adult mice (0.02 ml of 20% concentration in PBS), and such inoculated mice developed leukemia without any further treatment.

Control animals received an injection of 0.02 ml of PBS (the solution used for virus preparation) directly into one thymus lobe, instead of the virus passage.

Samples taken for histological studies were fixed in Bouin's fluid and stained with hematoxylin and eosin.

X-irradiation. Irradiation was performed with a General Electric Maximar 250 III (physical conditions, 230 kV, 15 ma,

[1] This work was supported by a grant from the Israel Cancer Association.

[2] Present address: Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Zürich, Switzerland.

[3] The abbreviations used are: APase, alkaline phosphatase; PBS, phosphate-buffered saline; *p*-NPP, *p*-nitrophenyl phosphate.

Received February 22, 1971; accepted July 6, 1971.

with 1-mm Al and 0.5-mm Cu filters; 50 cm; dose rate, 45 R/min).

Preparation of the Enzyme Extracts. Homogenizations and extractions of the tissues, as well as centrifugations, were done as previously described (6). In the 1st of the 2 time studies, only the Tris-HCl extraction step was performed. For each set of experimental measurements, 10 mice were sacrificed, and the particular tissues were combined and handled accordingly. All extractions and kinetic measurements were performed on the day that the mice were sacrificed.

Assay with *p*-NPP. The rates of enzymatic hydrolysis of *p*-NPP were determined as previously described (6). Standard assay mixtures contained 10^{-3} M *p*-NPP in 0.5 M Tris-HCl, pH 9.0. From plots of the spectrophotometric measurements, the velocities were determined, and the specific activities were calculated. The specific activity was defined as the M of *p*-nitrophenol formed per min per g of wet tissue per ml at room temperature.

The *p*-NPP was obtained from Sigma Chemical Company, St. Louis, Mo. All other compounds used were of analytical grade. Absorbance and pH measurements were made with a Zeiss Model PMQ spectrophotometer and a Radiometer Model 22 pH meter, with a Type G-202 B glass electrode and a Type K4312 calomel electrode or a Type GK3031 combined electrode, respectively.

RESULTS

Two sets of chronological time studies were carried out concerning the amounts of measurable APase activity during the development of lymphatic leukemia in various tissue homogenates.

APase Specific Activities Evaluated at Monthly Intervals. In the first experiment, lymphatic leukemia was induced by Virus Passage 127 inoculated into the thymus followed by 400 R of whole-body irradiation. One control group consisted of those with Virus Passage 127 injections into the thymus (to exclude the possible effects of mechanical injury to the thymus or enzyme activity).

The Tris-HCl-extractable APase levels of the thymus, mesenteric lymph nodes, spleen, liver, and kidney were measured at 30 and 60 days after virus or PBS injection into the thymus.

The results are summarized in Table 1. In the thymus, the APase specific activity remained essentially constant throughout the experiment in the 2 control groups that did not develop leukemia; namely, specific activity ranged from 0.6 to 1.2 in mice receiving Virus Passage 127 inoculation alone into the thymus and 0.5 in mice given injections of PBS. On the other hand, the APase specific activity in the thymus of the group treated with Virus Passage 127 followed by 400 R of whole-body irradiation (the group that developed a high incidence of lymphatic leukemia) increased. At 30 days after leukemia induction treatment, no leukemia development was observed in this group, and the APase activity in the thymus was similar to that observed in the control groups. At 60 days after virus inoculation, 60% of the treated mice developed lymphatic leukemia. The divisions of the tissues at the 60-day interval, according to gross diagnosis and organ weights (and further confirmed histologically), into leukemic and

Table 1

APase specific activities of various tissues from C57BL/6 mice at 30 and 60 days after inoculation of either PBS, Virus Passage 127, or Virus Passage 127 followed by 400 R of whole-body irradiation

For each set of experimental measurements, 10 mice were sacrificed, and the particular tissues were pooled.

Treatment	Tissue	APase specific activities	
		30 days after inoculation	60 days after inoculation
PBS	Thymus	0.5[a]	0.5
	Mesenteric lymph nodes	1.0	1.2
	Spleen	1.4	1.3
	Liver	2.1	1.6
	Kidney	27.6	11.0
Virus Passage 127	Thymus	0.6	1.2
	Mesenteric lymph nodes	2.7	0.7
	Spleen	4.7	5.0
	Liver	2.2	2.1
	Kidney	25.6	15.6
Virus Passage 127 followed by 400 R of whole-body irradiation	Thymus	0.7	7.6 (10.0)[b]
	Mesenteric lymph nodes	1.6	0.5 (20.0)
	Spleen	3.7	5.0 (11.1)
	Liver	1.7	3.0 (5.0)
	Kidney	16.0	36.0 (50.0)

[a] Values are M × 10^{-5} *p*-nitrophenol formed per min per g of wet tissue per ml at room temperature.

[b] Values in parentheses represent the specific activities of tissues divided on the basis of size; *i.e.*, enlarged and leukemic. In some cases, dissemination had occurred.

nonleukemic groups (10 mice in each group) were necessary to prevent the mixing of those tissues which were obviously leukemic and disseminated, from those which were not. The APase activity in the thymus at the 60-day interval was increased both in the nonleukemic (specific activity of 7.6) and leukemic (specific activity of 10) mice.

The APase specific activities in the other tissues (mesenteric lymph nodes, spleen, liver, and kidney) evaluated at the same time intervals were similar in the 3 tested groups as long as the mice were not diagnosed as leukemic. Minor fluctuations in the APase specific activities were noted in the different tissues taken from the control groups, but in no case was the fluctuation greater than the observed increase in the specific activities of the diseased tissues. In the leukemic mice, an increase in the APase specific activities was observed in all the tested organs.

APase Specific Activities Evaluated at Weekly Intervals. Because APase activity was only partially extractable in 0.1 M Tris-HCl, pH 8.0, and because the measurements at 1-month intervals were found to be too large, it was decided that an additional extraction step was necessary and shorter time intervals should be chosen. The extraction of the precipitate yielded by the first step (Tris-HCl, see "Materials and Methods") was suspended in H_2O, and a second extraction was performed with 1-butanol to a final concentration of 23% (v/v). This additional step resulted in a precipitate, after centrifugation, which was essentially free of APase activity as measured with *p*-NPP.

In the 2nd time study experiment, 2 different virus passage materials were used, namely Virus Passage 136 which is highly leukemogenic when injected into the thymus of adult C57BL/6 mice and Virus Passage 127 which yields a high incidence of lymphatic leukemia provided that the mice are exposed to irradiation shortly after virus inoculation into the thymus.

Mice were divided into 4 experimental groups and treated as follows: (*a*) inoculation of Virus Passage 136 directly into one thymus lobe of adult mice; (*b*) the matching control (injection of PBS directly into one thymus lobe); (*c*) inoculation of Virus Passage 127 into the thymus followed by exposure to 400 R of whole-body irradiation; and (*d*) the appropriate control group receiving PBS injection into the thymus followed by 400 R of whole-body irradiation.

The enzymatic analyses were performed at weekly intervals starting 2 weeks after virus or PBS injection into the thymus. The changes in the APase levels in the differently treated groups (10 mice from each group sacrificed weekly) were measured in the thymus, mesenteric lymph nodes, spleen, liver, and kidney. Due to the variability of the specific activities within each tissue from week to week, the results are expressed as SP/SP_0 ratios, where SP is the specific activity of the test group and SP_0 is the specific activity of the control; *i.e.*, PBS injected into the thymus was the control for Virus Passage 136, and PBS followed by irradiation served as the control for the Virus Passage 127 followed by whole-body irradiation. The results of the chronological changes in the Tris-HCl-extractable APase levels, as shown in Chart 1, indicate that only in the thymus does a significant increase occur over the time interval studied. This increase from zero or very little activity starts in the 7th week with Virus Passage

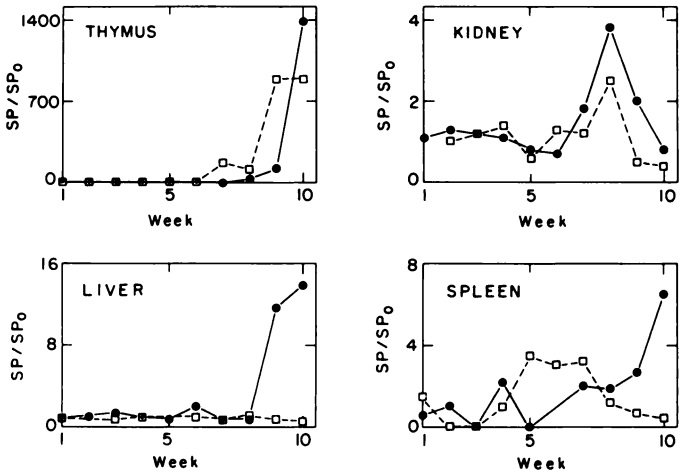

Chart 1. The changes in the Tris-HCl-extractable APase levels of the thymus, the kidney, the liver, and the spleen as a function of time (weeks) after inoculation of Virus Passage 136 (•) or Virus Passage 127 followed by 400 R of whole-body irradiation (□). The ratio of SP/SP_0 represents the APase specific activity of the test group (Virus Passage 136 or Virus Passage 127 followed by 400 R of whole-body irradiation), and SP_0 represents the specific activity of the control (PBS for Virus Passage 136 and PBS followed by irradiation for Virus Passage 127 followed by 400 R of whole-body irradiation).

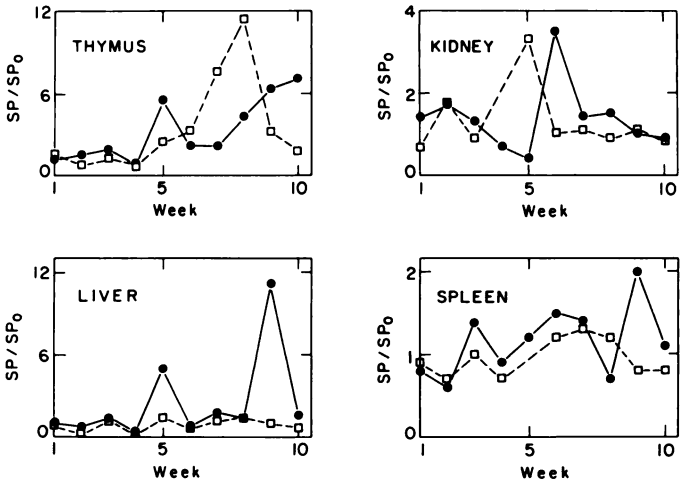

Chart 2. The changes in the butanol-H_2O-extractable APase levels of the thymus, the kidney, the liver, and the spleen as a function of time (weeks) after inoculation of Virus Passage 136 (•) or Virus Passage 127 followed by 400 R of whole-body irradiation (□). See Chart 1 for the definition of the SP/SP_0 ratio.

127 and in the 8th week with Virus Passage 136. In the other Tris-HCl tissue extracts, the ratios of the specific activities varied from week to week, and significant increases, such as in the liver with Passage 136, were not detected until dissemination had started. The results in Chart 2 for the butanol-H_2O-extractable activity are again variable, and only in the case of the thymus are increasing trends in the APase levels noted prior to lymphoma development (in the 5th week).

Histological examination indicated that, 9 weeks after virus inoculation into the thymus, 10% of the mice given injections of Passage 136 and 20% of those treated with Passage 127 plus

irradiation developed thymic lymphomas. At the 10th week, 30 to 35% of the treated mice in both groups were leukemic, and 1 week later the incidence increased to 50 to 70%. No significant difference in the mean thymus weight of the differently treated groups, sacrificed at weekly intervals starting 2 weeks after virus or PBS inoculation into the thymus, occurred during the first 9 weeks.

DISCUSSION

On the basis of the chronological study of the changes of APase levels during the development of lymphatic leukemia in C57BL/6 mice, induced by the radiation leukemia virus (Passages 127 and 136), a significant increase of APase activity was observed only in the thymus. As is apparent from Chart 1, the Tris-HCl-extractable APase activity of the thymus gave SP/SP_0 ratios, in both Virus Passage 136 and 127, of greater than 1000. In all other tissues, the maximum change of the SP/SP_0 ratio of the Tris-HCl-extractable activity was less than 10, except in the liver (Passage 136) and even then after dissemination had started. The extent of change of APase levels in all tissues is much less pronounced after butanol-H_2O extraction (Chart 2), and only in the case of the thymus was an increase in the APase activity noted prior to lymphoma development.

Histological evaluation indicated that the first lymphomas were observed 9 weeks after virus inoculation into the thymus (in both groups treated with either Virus Passage 136 or Virus Passage 127 followed by exposure to irradiation), and thereafter the increase in leukemia incidence was rapid and dissemination occurred in about one-half of the sick mice. Therefore, the increase of APase activity occurred prior to the neoplastic proliferation (Table 1 and Charts 1 and 2).

In these studies, the increase in APase activity was observed only in mice treated with Virus Passage 136 or in mice inoculated with Virus Passage 127 and further exposed to whole-body irradiation (both treatments yielding a high leukemia incidence). Inoculation of Virus Passage 127 into the thymus without further exposure to X-rays (a procedure inducing leukemia only in 10 to 20% of the injection-treated mice) did not affect the APase activity found in the normal thymus of C57BL/6 mice. This finding indicates that the virus itself [which was found to remain viable in the thymus for many months (1)] is not responsible for the increase in APase activity, but rather either the neoplastic transformation of thymocytes or the proliferation of the neoplastic cells is responsible. These observations coincide with the histochemical and histopathological studies of Lägerlöf and Kaplan (2), who showed that the development of APase activity is a specific response induced concomitantly with the neoplastic transformation, and these observations are contradictory to those of Siegler and Rich (7), who claim that the increase in APase activity occurs only after lymphoma cells proliferate and enlarge the thymus at least 3 times in weight. In the other tissues studied, significant increases in APase levels were observed only after dissemination, *i.e.*, after the 9th week.

A comparison of Charts 1 and 2 reveals that an elevation of the thymic APase level occurs at an earlier stage in the butanol-H_2O extract of the precipitate after Tris-HCl extraction. Experimentation is currently under way to clarify this observation.

ACKNOWLEDGMENTS

We thank Professor Ephraim Katchalski for his encouragement and suggestions in this work. We also gratefully acknowledge the technical help of Miss Shoshana Peller.

REFERENCES

1. Haran-Ghera, N. Influence of Host Factors on Leukemogenesis by the Radiation Leukemia Virus. Israel J. Med. Sci., *7:* 17–25, 1971.
2. Lägerlöf, B. A. M., and Kaplan, H. S. Specificity of the Relationship between Thymic Alkaline Phosphatase Activity and Lymphoma Development in Strain C57BL Mice. J. Natl. Cancer Inst., *38:* 437–458, 1967.
3. Lumb, J. R., and Doell, R. G. The Biochemical Characterization of Alkaline Phosphatase from Chemical- and Viral-induced Thymic Lymphomas of C57BL Mice. Cancer Res., *30:* 1391–1396, 1970.
4. Metcalf, D., Sparrow, N., and Wyllie, R. Alkaline Phosphatase Activity in Mouse Lymphoma Tissue. Australian J. Exptl. Biol. Med. Sci., *40:* 215–224, 1962.
5. Neumann, H., Wilson, K., and Haran-Ghera, N. Some Physical Properties of Alkaline Phosphatases Found in Various Tissues of AKR and C57BL/6 Mice, Normal and Leukemic. Federation European Biochem. Soc. Letters, *8:* 87–90, 1970.
6. Neumann, H., Wilson, K. J., Hauck-Granoth, R., and Haran-Ghera, N. A Comparative Biochemical Study of Alkaline Phosphatases in Normal and Leukemic Mice. Cancer Res., *31:* 1695–1701, 1971.
7. Siegler, R., and Rich, M. A. Significance of Increased Alkaline Phosphatase Activity in Viral-induced Thymic Lymphoma. Proc. Soc. Exptl. Biol. Med., *125:* 868–871, 1967.
8. Smith, C. Studies on the Thymus of the Mammal. XII. Histochemistry of the Thymuses of C57BL/6 and AKR Strain Mice. J. Natl. Cancer Inst., *26:* 389–404, 1961.