Cytochemical Changes in Lymph Nodes and Spleen of Rats after Total Body X-Radiation

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The effects of x-radiation on the chemical constituents of cells of the body are not well understood. Lymphoid tissue appears to be particularly sensitive to x-radiation, nitrogen mustard, and triethylene melamine. Aminopterin produces relatively little change in lymphoid tissue, but injures myeloid tissue severely. Few cytochemical studies of the effect of these agents on hemopoietic tissue have been undertaken.

The present study deals chiefly with the cytochemical alterations of the lymph nodes and spleen in the rat following treatment with x-ray.

Materials and Methods

The spleen and cervical lymph nodes from a total of one hundred twenty-seven young adult male albino rats (Harlan strain) were examined cytochemically. A group of fifty-six animals received a single exposure of 600 r total body radiation (250 kv. constant potential, 15 ma., 0.5 mm. Cu and 1.0 mm. Al, target distance 100 cm., 24.5 r/min.). The animals were placed at the periphery of a wooden wheel, 24 inches in diameter, which revolved in the beam at 1.3 rpm. In this experiment, a dose of 600 r total body radiation for a period of 26.5 minutes was found to be an approximately LD50/30. A minimum of six animals were sacrificed at each time interval following x-irradiation. The lymph nodes and spleen were removed at intervals of two, four, and twelve hours and one, two, four, eight, sixteen, and thirty-two days following x-radiation. Ten animals not exposed to x-radiation were used as controls.

Various chemotherapeutic agents including aminopterin (24 mg./Kg.), nitrogen mustard (methyl-bis (β-chloroethyl)amine hydrochloride) (1.8 mg./Kg.), and triethylene melamine (1.8 mg./Kg.) were also employed in dosage equivalents of an LD50 or greater for the rat. Each agent was prepared in saline immediately prior to administration of a single intraperitoneal injection. Thirty-four, twenty-nine, and eight animals receiving aminopterin, nitrogen mustard, and triethylene melamine, respectively, were sacrificed at various time intervals following injection and the spleen and cervical lymph nodes removed and examined cytochemically.

Tissues were fixed in 80 per cent ethyl alcohol for 14 hours, dehydrated and cleared (95 per cent and absolute alcohol and xylol, 1 hour each) and embedded in paraffin (2 changes, 3 hours each) at 57 C. Sections 4 μ thick were cut and mounted within 4 hours after the tissues had been embedded.

In the initial phase of this investigation, 80 per cent alcohol and Zenker-formol were used as fixatives. However, the critical time schedule of staining tissues compelled omission of Zenker-formol.

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of one fixative. A careful evaluation revealed 80 per cent alcohol to be satisfactory for most aspects of this investigation. Although 80 per cent alcohol is not an ideal morphologic fixative, it was possible to distinguish cell types in the lymph nodes and spleen with little difficulty (fig. 3).

Tissue sections were examined for the following substances: desoxypentosenucleoprotein was demonstrated by the nucleic reaction, and pentosenucleoprotein by ribonuclease digestion and toluidine blue technic. The periodic acid-Schiff reaction was used for mucopolysaccharides although other substances are colored by this reaction.

Protein-bound sulfhydryl groups were revealed by the diazo technic of Barnett and Seligman, and by the ferricyanide technic of Chévremont and Frédéric. The ferricyanide method is said to be nonspecific since many substances besides sulfhydryl compounds reduce ferricyanide. The diazo technic of Barnett and Seligman is considered to be the most specific for protein-bound sulfhydryl groups in tissue. Ethyl alcohol (80 per cent) was found to preserve adequately the thiol groups when compared with sections fixed in the trichloroacetic acid-alcohol recommended by Barnett and Seligman. A careful examination of both technics applied to sections of lymph node and spleen of the rat yielded comparable results. The only exception found was hemosiderin which reduced ferricyanide, but did not react by the diazo technic.

Alkaline phosphatase activity was studied by the Gomori technic. Tissues were placed in the alkaline phosphatase substrate solution exactly 24 hours after fixation and incubated at 37 C. for 18 hours in this solution. The slides were then washed thoroughly and placed in cobalt chloride for exactly 5 minutes, washed, and placed in dilute yellow ammonium sulfide for 2 minutes. Sodium glycerophosphate and 5-nucleotide were used as substrates, although both yield similar results in sections of the lymph node and spleen.

Certain objections have been raised to the alkaline phosphatase technic of Gomori. Long periods of incubation seem to produce diffusion artifacts, thus precluding true topographic localization of phosphatase activity. Coloration of the nucleus is considered to result from absorption of calcium phosphate by the nucleus, since biochemical studies reveal that the nucleus possesses only minimal alkaline phosphatase activity. In the present study, the over-all coloration of the cells by the Gomori technic was considered to be an indication of the alkaline phosphatase activity of the cell as a whole, with no attempt to distinguish cytoplasmic or nuclear reaction. Slides were incubated for periods of 15 minutes to 24 hours. The only difference noted after various times of incubation was an increase in the over-all intensity of the coloration of the whole section; no difference in localization within the section or within the cell was noted. An incubation period of 18
Plate I
(See legend, facing page.)
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hours was thus chosen as the most adequate period because the more intense reaction following such a long period of incubation allowed more precise evaluation of small differences in phosphatase activity. Reproducible results may be obtained by rigorously controlled experimental conditions and cytochemical procedures. It is possible to quantitate approximately on a plus-minus basis the cytochemical reactions by visual observation of the degree of stainability of the tissue.

OBSERVATIONS

X-radiation produced marked cytochemical changes in lymph nodes and spleen (figs. 1 to 6). These changes occurred very rapidly and preceded the time when structural alterations became discernible. The alkaline phosphatase reaction was diminished after x-radiation. The reactions of protein-bound sulfhydryl groups and pentosenucleoproteins were also decreased somewhat in the cells of the lymph nodes and spleen within two hours following irradiation. However, at twelve hours after exposure the cellular phosphatase reaction as well as the reaction of sulfhydryl groups and pentosenucleoprotein were more intense than in the nonirradiated cells. These reactions then remained increased until the fourth day after radiation, at which time the cells resembled untreated control tissues in respect to these elements. All cells of the irradiated tissues exhibited the qualitative changes described, although the intensity of the reaction differed in different types of cells.

Lymph Nodes

Lymphocytes of lymph nodes were particularly sensitive to x-radiation. Two to four hours following radiation, the lymphocytes exhibited marked diminution or complete loss of their phosphatase activity, changing from approximately ++ + + to zero or +. There was a complete loss in the reaction for pentosenucleoprotein, changing from + or ++ to zero; the reaction for sulfhydryl groups at four hours was slightly reduced, changing from about ++ to +. Morphologic signs of cellular degeneration and death (pyknosis, karyorrhexis, karyolysis) began between two and four hours and reached a maximum by twelve hours after x-radiation. Some lymphocytes exhibited a return of their cytochemical reactions and appeared to survive their dosage of x-radiation, since a few lymphocytes were found in all lymph nodes examined following radiation. No return of reactions for phosphatase, sulfhydryl or pentosenucleoprotein occurred in any of the lymphocytes that exhibited morphologic signs of degeneration. Those lymphocytes that appeared to be extremely sensitive to x-radiation exhibited in most instances irreversible cytochemical changes. Degenerating lymphocytes were phagocytized by clasmatocytes beginning within four hours, and this process was completed by twenty-four hours following irradiation. Phagocytized and extracellular nuclear fragments usually exhibited weak phosphatase reaction; the nuclear reaction appeared to increase in the degenerating nuclei due to the condensation of chromatin. Occasionally, lymphocytes undergoing dissolution were found to exhibit a few cytoplasmic granules that were larger and more strongly positive in their periodic acid-Schiff reaction than the granules of lymphocytes of normal untreated lymph nodes.22

The elongated reticular cells of the lymph nodes showed an initial decrease in the intensity of their phosphatase reaction (++ to zero, +) following x-radia-
tion. The intensity of reaction for pentosenucleoprotein (+ to zero) and sulf-
hydryl groups (+ + to +) was also diminished. In contrast to the majority of
lymphocytes, these transient cytochemical changes were apparently not lethal
for the elongated reticular cells. The initial decrease in the cytochemical reaction
of the present series of tissues was followed by an increase in the reaction for
pentosenucleoprotein (+ +), sulphydryl groups (+ + + +), and alkaline
phosphatase (+ + + +) until the fourth day when the intensity of reaction
returned to control levels. Concomitant with the increase in these reactions oc-
curring in the elongated reticular cells, the size and number of these cells in the
cortex of the lymph node increased (figs. 3, 4). During the first to fourth day
following x-radiation, an increase was observed in the reactions for alkaline
phosphatase, pentosenucleoprotein, and sulphydryl groups of the nucleoli of the
elongated reticular cells and lymphoblasts, in addition to an increase in the size
of the nucleoli.

The qualitative changes in the cytochemistry of the plasma cells and clasmato-
cytes following x-radiation were similar to the changes described for the lympho-
cytes and elongated reticular cells. The alkaline phosphatase, pentosenucleo-
protein, and sulphydryl reactions of the plasma cells changed from + + + or
+ + + + to + + immediately following irradiation, then increased to + + + +
by the first day and returned to control levels by the fourth day. The phospha-
tase, sulphydryl, and pentosenucleoprotein reactions of the clasmacytos changed from + or + + to zero or + within two hours after radiation and re-
turned to control levels or perhaps slightly greater than control levels by the
end of the first day.

Cytochemical and morphologic changes occurred in the walls of the small
blood vessels in the lymph node, particularly the small arterioles in the cortical
region of the lymph node. The endothelial cells of these vessels exhibited a de-
crease in reaction for alkaline phosphatase, sulphydryl groups, and pentosenucleo-
protein. Morphologically, the endothelial cells appeared to be shrunken, the
nucleus was smaller, and the chromatin more condensed than in nonirradiated
endothelial cells. The cytoplasm was granular, somewhat vacuolated, and ir-
regular in shape. These changes were evidently transient, for the vessels appeared
to have re-established their normal features by the second day after radiation.
The blood vessels of the cortex of the lymph node seemed to have proliferated
during stages of recovery.

Spleen

The spleen of irradiated rats was reduced in size with decrease in the amount
of white pulp and increase in red pulp. The white pulp exhibited cytochemical
and morphologic changes similar to those previously described for the cortex of
the lymph node. The number of lymphocytes in the red pulp was reduced and
the clasmacytos increased. The clasmacytos were richer in alkaline phos-
phatase and sulphydryl groups for sixteen days after irradiation, in contrast to
the clasmacytos of the cortex and medulla of the lymph node and white pulp
of the spleen, both of which showed only transient increase in clasmacytos dur-
ing the first twenty-four hours. The clasmacytos of the red pulp showed an
increase in erythrocytic phagocytosis and an increase in hemosiderin which
changed from + to +++ within four days following exposure. Erythrocye phagocytosis in the lymph nodes was slight. The observed increase in the phosphatase and sulfhydryl reactions in the spleen following irradiation was apparently due to the relative increase in the red pulp and the number of clasmocytes as well as an actual increase in the cytochemical reactions within individual clasmocytes. Biochemical studies indicate an increase in alkaline phosphatase activity (with glycerophosphate and 5-nucleotide) in the spleen of the mouse following x-radiation.

Antileukemic Chemical Agents

Cytochemical observations upon the cervical lymph nodes and spleen of the rat following a single injection of aminopterin, nitrogen mustard, and triethylene melamine have revealed that no apparent cytochemical changes occur prior to discernible morphologic alterations. In this experiment, triethylene melamine produced more damaging effects upon the lymph nodes and spleen than did nitrogen mustard; very few changes were noted either morphologically or cytochemically following aminopterin, although bone marrow destruction appeared to be extensive. Lymphocytes were the only cellular element of these organs which appeared to be damaged by these chemical agents. Lymphocytes undergoing degeneration exhibited a decrease in their reactions for pentosenucleoprotein, alkaline phosphatase, and sulfhydryl groups. However, many lymphocytes exhibited neither morphologic nor cytochemical signs of degeneration. Clasmocytes showed only a transient increase in number and actively phagocytized degenerating lymphocytes. Elongate reticular cells did not appear to be injured by the antileukemic chemical agents; in fact, between one and four days following the injection of nitrogen mustard or triethylene melamine these cells increased in number and size and exhibited concomitantly an increase in the intensity of the reactions for pentosenucleoprotein, alkaline phosphatase, and sulfhydryl groups. No other cytochemical alterations were observed in any of the other cellular elements of the lymph nodes and spleen following treatment with these chemical agents.

Discussion

Cytochemical changes preceded morphologic alterations in the cells of the lymph nodes and spleen following x-radiation. The cytochemical changes occurred very rapidly and consisted of a decrease in the intensity of reactions for pentosenucleoprotein, protein-bound sulfhydryl groups, and alkaline phosphatase. Although these alterations were essentially similar in all cells of the lymph node and spleen, lymphocytes appeared to be more susceptible to injury than other cells of these organs. A very large proportion of lymphocytes and some plasma cells exhibited an irreversible decrease in intensity of their reactions for pentosenucleoprotein, sulfhydryl groups, and alkaline phosphatase followed by morphologic dissolution. Cells of the lymph nodes and spleen which revealed no signs of degeneration exhibited a transient decrease followed by an increase in the intensity of reactions for alkaline phosphatase, sulfhydryl groups, and pentosenucleoprotein. No changes were observed in the nucleal or periodic acid-Schiff reactions following x-radiation.
There is no correlation of the degree of cytoplasmic staining for pentosenucleoprotein of the cells of the lymph nodes and those of the spleen. It would seem that the concentration of pentosenucleoprotein was not the major factor per se in determining all sensitivity to irradiation.

Following x-radiation, the lymphocytes, elongated reticular cells, and clasmatocytes reacted differently in comparison to lymphoblasts and plasma cells. The former showed only weak reaction for pentosenucleoprotein, whereas the lymphoblasts and plasma cells were rich in pentosenucleoproteins. Following x-radiation, the number of lymphocytes markedly decreased and the plasma cells increased initially, while the elongated reticular cells and clasmatocytes increased. Lymphoblasts and many of the elongate reticular cells appeared to transform into clasmatocytes soon after x-radiation.

The lymphocytes that exhibited the most marked change in their phosphatase reaction (++++ to zero or +) were readily destroyed by x-radiation, while elongated reticular cells appeared to be radioresistant and showed only a slight change in their phosphatase reaction (++ to zero or +). This observation differs from that of Doyle who observed that the radioresistant reticular cells of the caecum (appendix) of the rabbit exhibited relatively greater alkaline phosphatase reaction than that of the lymphocytes. He believed that the lymphocytes were responsible for only a minor portion of the total phosphatase reaction in the caecum. Using the 18 hour incubation time of the present investigation, lymphocytes appeared to be important in the total phosphatase reaction of the lymph nodes of the rat. Doyle noted little or no change in the over-all phosphatase reaction of the caecum of the rabbit at intervals of eight hours, and five, eleven, and twenty-four days following an exposure to 625 r total body radiation. In contrast to Doyle's studies, the results of the present investigation suggest that striking changes in the phosphatase reaction occur within two hours, and that the over-all reaction of the lymph nodes and spleen returns to and may exceed control levels within twelve hours after x-radiation. The majority of lymphocytes degenerated and exhibited an irreversible decrease in their phosphatase reaction, while the elongated reticular cells and a few lymphocytes exhibited a reversible phosphatase reaction with an initial decrease followed by an increase in the reaction. These observations suggest that cells that exhibited the greatest change in their alkaline phosphatase reaction immediately following x-radiation may be the most sensitive to irradiation.

A marked and relatively prolonged decrease in the phosphatase and sulfhydryl reactions in the cells of the lymph node and spleen suggest an impairment of cellular metabolism, indicating damage to the cell and impending cell death if metabolism is extensively altered. An increase in the phosphatase, sulfhydryl, and pentosenucleoprotein reactions appears to reflect an increase in the metabolism of the cell, coinciding with cellular regeneration and proliferation following x-radiation.

The morphologic changes observed in the lymph nodes and spleen following treatment with x-radiation, aminopterin, nitrogen mustard, and triethylene melamine were in agreement with the observations described by other investigators. Cytochemical changes were not as great following treatment with the chemical agents as occurred following x-radiation with the dosages given. No
cytochemical changes were observed prior to morphologic changes following treatment with the chemical agents although cytochemical differences did occur before morphologic alterations became apparent following x-radiation.

Histochemistry has entered a phase characterized by critical analyses of the specificity and validity of various methods employed for the localization of certain chemical entities in cells and tissues. A few of these points have been indicated for the methods employed in this investigation. Differences have been observed in the reactivity of the cells of the lymph node and spleen following x-radiation using histochemical methods. Although an actual increase or decrease in specific substances or enzymatic activities may be questioned it is obvious that some change or changes must occur in these tissues in order to account for the differences in the well-controlled histochemical reactions.

**Summary**

Marked cytochemical changes were observed in the cells of the lymph nodes and spleen of one hundred and twenty-seven albino rats observed over periods of two hours to thirty-two days following a single dose of 600 r total body x-radiation. The intensity of the cytochemical reactions for pentosenucleoprotein, protein-bound sulphydryl groups, and alkaline phosphatase decreased within two hours after x-radiation. This decrease in the cytochemical reactions occurred very rapidly and preceded any discernible morphologic alteration. Lymphocytes that were extremely sensitive to x-ray exhibited most instances irreversible cytochemical changes. The cells that survived the lethal effects of x-radiation exhibited an increase in intensity of the reactions for pentosenucleoprotein, protein-bound sulphydryl groups, and alkaline phosphatase between twelve hours and four days following irradiation. The reactions for deoxypentosenucleoprotein and mucopolysaccharides were unaltered in the cells of the lymph nodes and spleen following x-radiation.

**Summary in Interlingua**

Marcate cambiamenti cytochimic esseva observate in le cellulas del nodos lymphatic e del splen de 127 rattos albin durante periodos de inter duo horas e 32 dies post un dose unie de 600 r de radios X administrate al integre corpore. Le intensitate del reactiones cytochimic pro pentosenucleoproteina, gruppos de sulphydrylo ligate a proteina, e phosphatase alcalims decresceva intra duo horas post le irradiation. Iste diminutio,s in le reactiones cytochimic occurreva rapidissimemente e precedeva ulle discernibile alteration morphologic. Le lymphocytes, que esseva extremely sensibile a radios X, exhibiva generalmente irreversibile cambiamentos cytochimic. Le cellulas que superviveva al effectos letal del radios X exhibiva un augmento in le intensitate del reactiones pro pentosenucleoproteina, gruppos de sulfydrylo ligate a proteina, e phosphatase alcalim durante periodos de inter dece-duo horas e quatro dies post le irradiation. Le reactiones pro disoxypentosenucleoproteina e mucopolysaccharidos in le cellulas del nodos lymphatic e del splen non esseva alterate per le irradiation.

**REFERENCES**

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