Neoplasm Studies

XI. The Effects in Tissue Cultures of N,N,N',N'-Tetramethyl-*o*-Phenylenediamine and Other Compounds on Malignant Lymph Nodes*

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These experiments were conducted on the basis of recent unpublished findings by Kensler that the respiration of malignant lymphoid tissue is inhibited by tetramethyl-*o*-phenylenediamine while that of its normal counterpart is modified only slightly if at all. The investigations described in this report, which is the third of a series of viability experiments (1, 2), were planned to determine the effect of these and other compounds on the viability of lymphocytes from a variety of lymphoid tissues grown in tissue culture. The cultures of human material were of normal lymph nodes, lymphosarcoma, lymphatic leukemia, and nodes of Hodgkin's disease. Cultures were made also of normal rat lymph nodes and a transplantable rat lymphosarcoma (3). The studies reported are based on over 1,000 cultures, totalling about 3,000 fragments of human material and about 400 cultures of rat tissues.

For the controls the tissue culture medium consisted of fowl blood plasma, 1 part; homologous serum, 3 parts; chick embryonic extract, 1 part.

Immediately after explantation the cultures frequently showed an appreciable halo of cells about the explant, a condition to be distinguished from an active outwandering of cells. A few leukocytes might appear after 3 or 4 hours of incubation, while the lymphocytes began to migrate in increasing numbers after 8 to 10 hours.

The majority of the lymphocytes from normal nodes were of the small, round type, interspersed with a few of the intermediate and an occasional example of the large type. Cultures of the neoplastic nodes presented the typical appearance of lymphocytic outgrowths, except for the presence of considerable cellular debris, a greater variety of shapes and sizes of the outwandering cells, and frequency of the large type of lymphocytes.

In the majority of cases, suitable outgrowths for experimentation developed after 48 hours of incubation, while some attained sufficient proportions at 24 hours. Others were 24 to 48 hour growths from subcultured explants of fragments started a week or two earlier.

The compound to be tested was introduced into a growing culture by adding a medium in which the serum had been replaced by Tyrode solution or serum, containing a given concentration of the compound. The figures given below are those of the final concentrations.

The compounds employed were sodium malonate, hydroxyquinoline, rotenone, N,N-dimethyl-*p*-phenylenediamine, N,N,N',N'-tetramethyl-*o*-phenylenediamine, and N,N,N',N'-tetraethyl-*o*-phenylenediamine.

Sodium malonate was tested in concentrations from 0.01 to 0.05 *M*. The concentration of 0.05 *M* was found to be toxic in a few hours to all types of cells. It showed no differential action between lymphocytes and other wandering cells whether from normal or neoplastic tissues, including nodes from Hodgkin's disease. A concentration of 0.03 *M* had no toxic action during 48 hours of exposure.

8-Hydroxyquinoline, in a concentration of 0.2 saturation in serum, likewise showed a general toxic effect within 24 hours on cells of both normal and neoplastic origin but to a less pronounced degree. The effect on Hodgkin's nodes was not tested. Weaker concentrations (0.1 and 0.05 saturated) were nontoxic throughout.

Rotenone, in a concentration of 0.2 saturation in serum, was also found to be nonspecific in its toxic effect, which became evident only after 48 to 72 hours. A concentration of 0.1 saturation was definitely less effective but still nonspecific. An exception appeared to be the lymph nodes of Hodgkin's disease, the lymphocytes of which were slightly more resistant than those from normal nodes or from lymphosarcoma.

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N,N-Dimethyl-p-phenylenediamine, in a concentration of 0.001 *M,* was toxic to the lymphocytes but not to the macrophages and fibroblasts in cultures of all types of tissues used. A large proportion of the lymphocytes underwent complete degeneration within 5 to 6 hours, while the macrophages and fibroblasts were only slightly, if at all, affected. The macrophages tended to round up and become inactive but a few hours later recovered completely, although they developed yellow vacuoles which eventually became pink. The fibroblasts remained normal throughout. The fat drops of adipose cells acquired a pronounced and persistent red-orange color. The same coloration was evident in extracellular fat drops of older cultures. At a concentration of 0.004 *M* all the cells, including fibroblasts and macrophages, disintegrated within 24 hours.

Cells from the nodes of Hodgkin's disease reacted in a similar fashion except that the macrophages, Dorothy Reed cells, and fibroblasts became granular during a brief inactive period but subsequently recovered.

For the lymphoid cells of the neoplastic tissues the critical concentration in general was 0.001 *M,* lower than that for the normal tissues, which was 0.0075 *M.* The lymphosarcomas showed a slight variability in their reaction, the toxic level varying in different tumors between 0.001 and 0.0025 *M.* The toxic effect on the cells was first made evident by retraction of their pseudopodia. Granulation of the cytoplasm steadily increased until the final stage of complete disintegration was reached. All stages up to disintegration were reversible. In borderline concentrations short of the toxic dose, the lymphocytes frequently appeared unhealthy after a sojourn of 5 to 6 hours in the experimental medium. However, after 24 hours they had resumed their normal appearance and activity. In the higher toxic concentrations of 0.01 and 0.05 *M* the lymphocytes did not undergo disintegration but were killed by fixation within a few hours. The nuclei of these fixed cells frequently acquired a pink color.

TABLE I: EFFECT OF N,N,N',N'-TETRAMETHYL-*o*-PHENYLENEDIAMINE ON WANDERING CELLS IN CULTURES OF HUMAN LYMPHOID TISSUES

Molar concentrations	Macrophages, fibroblasts, normal and from neoplasms	Lymphoid cells from nodes of		
		Hodgkin's disease	Lymphosarcoma, lymphatic leukemia	Normal subject
0.05	Toxic	Toxic	Toxic	Toxic
0.01	Nontoxic	"	"	A few survivals
0.0075	"	"	"	Nontoxic
0.005	"	"	"	"
0.0025	"	A few survivals	"	"
0.001	"	Nontoxic	A few survivals	"

N,N,N',N'-Tetramethyl-o-phenylenediamine. — The results of exposure to this compound are summarized in Table I. The macrophages and fibroblasts in cultures of normal and malignant tissues were equally unaffected by exposure to concentrations up to and including 0.01 *M* of the compound. With time the cells gradually acquired yellow inclusion bodies. Occasionally, after exposures of about 48 hours to concentrations of 0.01 *M,* the macrophages developed pink vacuoles. The difference between the normal and neoplastic tissues appeared in the reactions of the lymphocytes.

For lymphocytes of normal lymph nodes the critical concentration was 0.0075 *M.* In this concentration some of the lymphocytes showed a retraction of their pseudopodia and an infrequent increase in granulation of the cytoplasm. These changes were temporary, the lymphocytes returning to their normal appearance within a few hours. Disintegration occurred only very rarely. A concentration of 0.005 *M* exerted no effect whatsoever.

The sensitivity of lymphoid cells from the nodes of Hodgkin's disease was similar to that of the lymphosarcoma. In concentrations of 0.005 and 0.0075 *M* they were invariably destroyed, either by disintegration or by coagulation, the nuclei becoming pink. The Dorothy Reed cells were more resistant, resembling in this way the macrophages except for a tendency to become highly granular and vacuolated. In common with the macrophages and fibroblasts, the Dorothy Reed cells developed yellow inclusions after an exposure of 24 hours or longer.

The rat lymphosarcoma and normal lymph nodes grew excellently in the cultures. Their growth was more uniform than that of the human material and their sensitivity to the toxic compounds used was more consistent. This uniformity in growth and reaction, in contrast to that of the human lymphosarcomas, may be due to the controlled source of supply in the experimental rat.

N,N,N',N'-Tetraethyl-o-phenylenediamine was tested on cultures of human neoplastic nodes. Its action was similar except for its lesser toxicity, the critical toxic concentration being 0.005 *M,* or double that for the tetramethyl compound.

DISCUSSION AND SUMMARY

The compounds tested on normal and neoplastic lymphoid tissues may be divided into three groups, according to their action on the outgrowing cells of the cultures.

In the first were those the minimum effective concentration of which indiscriminately destroyed the cells. These were sodium malonate, 0.005 *M;* 8-hydroxyquinoline, 0.2 saturation in serum; and rotenone, 0.1 saturation in serum.

In the second group was N,N-dimethyl-*p*-phenylenediamine which, at a concentration of 0.001 *M,* destroyed all lymphoid cells irrespective of their source but did not affect the macrophages and fibroblasts. A concentration sufficient to destroy macrophages and fibroblasts was 0.004 *M.*

In the third group were N,N,N′,N′-tetramethyl-*o*-phenylenediamine and N,N,N′,N′-tetraethyl-*o*-phenylenediamine. Both resembled N,N-dimethyl-*p*-phenylenediamine in exerting a more drastic action on lymphocytes than on macrophages or fibroblasts. On the other hand, they were definitely more toxic to the lymphoid cells of the neoplastic than to those of the normal lymph nodes.

The critical toxic levels for lymphoid cells of the various cultured tissues to tetramethyl-*o*-phenylenediamine were found to be as follows:

Normal lymph nodes (human and rat)	0.0075 *M*
Hodgkin's disease	0.0025 *M*
Lymphosarcoma (human and rat)	0.001–0.0025 *M*
Leukemic node (human)	0.001 *M*

The closely related tetraethyl-*o*-phenylenediamine was tested only on human lymphosarcoma and leukemic nodes. It was found to have a similar specific toxicity for lymphocytes but with a potency of about half that of tetramethyl-*o*-phenylenediamine.

It is not possible from present data to decide upon the actual identity of the lymphoid cells of the normal and malignant tissues studied. The majority of the cells appearing in cultures of normal lymph nodes are of the small round cell type, but those of similar appearance in malignant nodes may not be precisely those of the normal nodes. This is suggested by greater variety in the shape of cells in malignant cultures and their tendency to form irregularly lobate instead of the characteristic relatively minute and narrow pseudopodia of the normal lymphocytes. They may be of an immature type, which raises the possibility that immaturity *per se* is the causative factor in their excessive sensitivity. However, the over-all difference in sensitivity of lymphoid cells from malignant and normal sources to tetraethyl- and tetramethyl-*o*-phenylenediamine is sufficiently pronounced to be significant. It supports the findings of Kensler in his respiration studies.

REFERENCES

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