Eosinophilic Response in Glioblastoma Tissue Culture after Addition of Autologous Lymphocytes

Abstract. *In experiments designed for the study of interactions in vitro between the cells of glioblastoma multiforme and lymphocytes from the same donor, a marked eosinophilic response was noted in three out of six cases. This was an entirely unexpected finding since few, if any, eosinophils survive in tissue cultures. These cells incorporated ^{3}H-cytidine and ^{3}H-leucine 72 hours after the cultures had been established, an indication that they were metabolically active.*

To obtain evidence concerning the immunologic aspects of human glioblastoma multiforme, we have studied the interaction in vitro between neoplastic cells and lymphocytes from the same donor. We now report the occurrence of significantly increased concentrations of eosinophils in these mixed cultures.

Fresh tissue from six cases of biologically active glioblastoma multiforme was minced with scissors and washed in balanced salt solution. Small tumor fragments were explanted on cover slips (30 by 10 mm) and cultured at 37°C (*1*). After 7 to 12 days, cultures with abundant growth were selected for further study. Ten milliliters of heparinized venous blood was withdrawn from the tumor donor, and the lymphocytes were separated from it (*2*). The separated cells were washed, centrifuged twice, and resusupended in culture medium. The number of cells was adjusted in a counting chamber to 400,000 ± 10 percent per milliliter. Several smears were prepared from the suspension and stained with Giemsa solution for differential count. The following types of cultures were established: (i) tumor cells, (ii) tumor cells with lymphocytes, (iii) lymphocytes, and (iv) lymphocytes with phytohemaglutinin (*3*). Each type was cultured in at least 12 Leighton tubes. The number of tumor cells varied from 700 to 900 cells ± 15 percent per slide, and the lymphocytes were kept at 400,000 cells ± 10 percent per tube. After incubation for 72 hours at 37°C, the cultures were exposed to tritiated cytidine or tritiated L-leucine for 2 hours (*4*). The slips with the attached cells and also smears made from centrifuged cell deposits were coated with Kodak NTB-3 emulsion by the technique of Kopriva and Leblond (*5*), except the emulsion was diluted to one part in two parts of 1 percent aqueous filtered commercial detergent solution. The autoradiographs were developed after a 2-week exposure, and the slides were stained with Giemsa solution.

Table 1 gives a summary of all cell counts. No preparation showed more than 0.1 percent monocytes, and these were not counted. Contamination with erythrocytes was insignificant; their number ranged from 10 to 15 per 1000 cells, except for case 6 where there were 80 erythrocytes per 1000 cells after the separation. Again, these elements were not scored. The admixture of eosinophils was highest in case 6, amounting to about 2.5 percent. No healthy-appearing polymorphonuclear neutrophils were present in cultures terminated after 72 hours. The predominant cell population comprised small and "transformed" lymphocytes, generically termed mononuclear. In three series of cultures of tumor cells and lymphocytes (cases 2, 3, and especially 6), a significant increase in the number of eosinophils was noted. Those were aggregated in high concentration around the tumor explant and between tumor cells (Table 1). At the periphery of a tumor explant the percentage of eosinophils was much lower, and in areas remote from cell growth in the tumor only a sporadic eosinophil could be seen. Roughly one-half of the eosinophils in the cultures were adult cells with two pouch-shaped nuclear segments. About 20 percent were juvenile forms with indented, but not yet segmented, nuclei (Fig. 1a), and 20 percent were immature cells with ovoid, or round, excentrically placed nuclei (Fig. 1, b and c). Ten percent of the eosinophils could be classified as pathologic forms with three or more nuclear segments or with signs of degeneration (Fig. 1d).

In both cases 3 and 6, 96 percent of the eosinophils had a significant uptake of cytidine (Table 2, Fig. 1), an indication of RNA synthesis. Cells close to or remote from tumor cells did not differ with respect to labeling with ^{3}H-cytidine. Only 8 percent of the eosinophils close to the tumor explant were significantly labeled with ^{3}H-leucine in the cytoplasm; in remote areas 3 percent were labeled. In these experiments the background was less than four grains per 1000 μ^2, and only cells with five or more grains were scored as labeled.

The presence of a high concentration of eosinophils in 72-hour-old cultures of neoplastic cells and lymphocytes is most unusual as the eosinophils rarely survive more than 24 hours in vitro (*6*). This fact was confirmed with the control cultures (Table 1). The initial differential counts make clear that the eosinophils increased in numbers. If we assume that they were derived from some cellular elements present in the culture, which is indicated by the presence of immature forms, then it is interesting that lymphocytes have been

Table 1. Cell populations before and after 72 hours of culturing. All cell counts given are sums of 8 to 12 replicate cultures from each sample. Between 500 to 750 cells were counted in each sample. Differences between separate cultures were not significant and did not affect the mean count. All lymphocytes with a diameter of 9 μ or more were classified as "large."

Case	Eosinophils in blood (%)	Cell population after separation and before culture				Cell population at 72 hours							
						Tumor cells and lymphocytes				Lymphocytes with phytohemaglutinin		Lymphocytes only	
						Area close to tumor explant		Area remote from tumor explant					
		Small lymphocytes	Large lymphocytes	Polymorphonuclear	Eosinophils	Mononuclear	Eosinophils	Mononuclear	Eosinophils	Mononuclear	Eosinophils	Mononuclear	Eosinophils
1	3	5068	607	232	93	5943	57	5950	50	6000	0	6000	0
2	0	5221	579	197	3	5816	184	5946	54	5973	27	5982	18
3	5	5426	312	260	2	5669	331	5913	87	6000	0	5998	2
4	1	5369	394	162	58	5998	2	5963	37	6000	0	6000	0
5	3	5100	407	415	88	6000	0	6000	0	6000	0	6000	0
6	4	5051	586	216	147	5272	728	5718	282	5966	34	5976	24

Table 2. Distribution of eosinophils labeled with ^{3}H-cytidine. Background less than four grains per 1000 μ^2. Cells with five or more grains were scored as labeled. The vast majority of cells had grain counts of 15 or more. All counts are expressed in percentage and are based on 100 cells counted.

Case	Nucleus only	Cytoplasm only	Nucleus and cytoplasm	Not labeled
3	56	2	38	4
6	60	4	34	2

suggested as a potential stem cell for eosinophils, though no experimental proof has been offered (*7*). When lymphocytes were cultured without specific antigen for 72 hours (Table 1), we found eosinophils only in 12 out of 72 cultures, and then their concentration never exceeded 0.5 percent. Moreover, only an ocacasional eosinophil incorporated ^{3}H-cytidine, and none was labeled with ^{3}H-leucine. Also, in cultures of lymphocytes incubated with precursors of RNA and protein immediately after separation, labeling of whatever eosinophils were present was minimal. These considerations suggest that the enrichment of eosinophils in the cultures of combined neoplastic cells and lymphocytes resulted from an active proliferation of this cell type.

The significance of the observed eosinophilic response remains obscure.

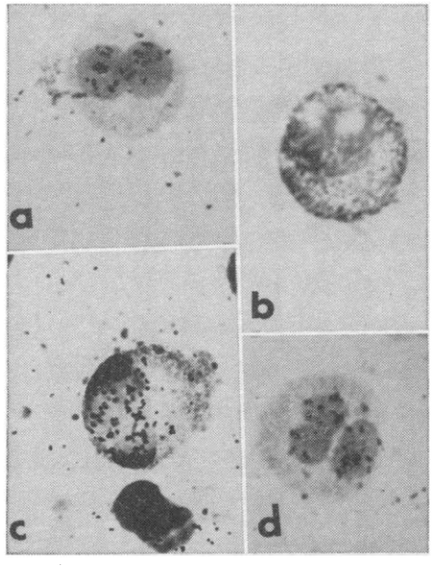

Fig. 1. Eosinophils from cultures of mixed tumor cells and lymphocytes stained with Giemsa (× 1200). (a) Juvenile form labeled with ^{3}H-cytidine (case 3). (b) Immature form with ovoid nucleus. No uptake of ^{3}H-leucine (case 3). (c) Immature form with ovoid nucleus labeled with ^{3}H-cytidine (case 3). (d) Abnormal form with three nuclear segments labeled with ^{3}H-cytidine (case 2).

Eosinophils play an important role in immune reactions in vivo (*8*); however, the exact function of these cells is not clear. Our observations could support the assumption that eosinophils participate in immune reactions involving tumor cells and autologous lymphocytes, because in all mixed cultures transformation of lymphocytes into "blast-like" forms was observed; the incidence of these forms ranged from 4 percent to 11 percent. This finding indicates an immune reaction in which the lymphocytes respond by transformation to "recognized" neoplastic antigenic groupings to which they had been sensitized while circulating (*9*). The fact that a significant eosinophilia in the mixed cultures occurred only in three out of six cases would indicate that such a response is not an essential factor in the interaction between tumor cells and autologous lymphocytes in vitro. It is noteworthy that we have seen marked eosinophilia in sediments of spinal fluid from patients with intracerebral neoplasms, including one case of glioblastoma multiforme. This fact suggests that an eosinophilic response to neoplastic processes is not restricted to a milieu in vitro (*10*).

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References and Notes

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3. Bacto Phytohemaglutinin M, Difco, in a concentration of 0.1 ml of rehydrated PHA in 10 ml of culture medium.
4. ^{3}H-Cytidine (specific activity, 5.87 c/mmole) and L-leucine-4, 5-^{3}H (specific activity, 60.0 c/mmole) from New England Nuclear Corp. at a concentration of 2 μc/ml of culture medium.
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Zeatin and Zeatin Riboside from a Mycorrhizal Fungus

Abstract. *The puffball fungus* Rhizopogon roseolus *produces and releases three cytokinins when it is cultured in liquid media. Two of these compounds have been isolated in crystalline form from such media and were found to have properties identical to those of synthetic zeatin and zeatin riboside.*

Zeatin, a substituted purine which belongs to the group of plant growth substances known as cytokinins, is extremely effective in promoting cell division in plant tissue cultures (*1–3*) and also displays activity in various (*4*) developmental tests. The compound and its ribose and ribosephosphate derivatives occur in young maize kernels (*1, 3, 5, 6*), and the free form has been isolated from plums (*2*). The picrate derivative of zeatin (*1*) and the barium salt of the monophosphate nucleotide have been obtained in crystalline form (*6*). My report is concerned with the production of cytokinins by the mycorrhizal fungus *Rhizopogon roseolus* (Cda.) Th. Fr. (*7*), the isolation of a substituted purine base and its riboside in crystalline form from media in which the fungus was grown, and identification of the compounds as zeatin and zeatin riboside.

The idea that mycorrhizal fungi might produce cytokinins came from the realization that the hypertrophy of root cortex cells that is common to ectotrophic mycorrhizal associations bears some resemblance to the hypertrophy that may result in cortex cells when roots are treated with kinetin, another cytokinin (*8*, for example). *Rhizopogon roseolus* forms mycorrhizal associations with trees such as pines, and assays for cytokinins in liquid media in which this fungus had been growing revealed a high level of cytokinin activity. Soybean cells (var. Acme) that do not grow in the absence of cytokinins (*9*) were used in the assay. Paper chromatography of the filtrate and subsequent assay with the soybean tissue showed that most of the cell division activity was located at the positions to which synthetic zeatin and its riboside migrated. A third active component was detected in much smaller amounts at the position where zeatin ribose-monophosphate would be expected. The activity was therefore tentatively assumed to be due to cytokinins of the substituted purine type, and an isola-