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29 January 1973

Rejection of Tumor Cells in vitro

Abstract. *The emergence of lymphoblast-like cells, capable of rapidly destroying tumor cells, was observed in primary cultures of an antigenic sarcoma transplantable in strain 13 guinea pigs. It is likely that these cytotoxic cells represent the progeny of lymphocytes sensitive to tumor antigens that had infiltrated the tumor tissue.*

Although the weight of experimental evidence indicates that most tumors are antigenic and elicit an immune response in the host, in only a few cases is the tumor rejected after the onset of tumor growth, and most tumors progress until the host's death (*1*). A number of workers have shown that some tumors are infiltrated with lymphocytes and other types of leukocytes and have ascribed to these cells a role in the host's resistance to the tumor (*2*). We report here that tumor-reactive lymphocytes infiltrating a solid tumor could outgrow the tumor cells in primary cultures through a cytotoxic reaction against the tumor cells.

The tumor used in these studies was a strongly antigenic methylcholanthrene-induced sarcoma D (MC-D), transplantable in inbred Sewall Wright strain 13 guinea pigs (*3*). For the preparation of cell suspensions, nonnecrotic pieces of MC-D were treated with 0.25 percent trypsin (Difco) in spinner-modified Eagle's medium (Difco) at room temperature for 30 minutes. The released cells were collected, sedimented (400*g*, 4°C, 10 minutes), and resuspended at 10^5 to 10^6 cells per milliliter in the complete medium described below. The cells were then cultured in Eagle's minimal essential medium that had been supplemented with nonessential amino acids (Difco) and 200 ml of heat-inactivated fetal calf serum (Gibco) per liter of medium. Penicillin and streptomycin (Difco) were added to final concentrations of 100 unit/ml and 100 μg/ml, respectively; the final medium is referred to as the complete medium (CMEM). All cultures were incubated at 37°C in a water-saturated atmosphere containing 5 percent CO_2. Cell lines of MC-D cultured for 40 days were able to form tumors on reinjection into syngeneic guinea pigs, resulting in death of all animals within 50 days.

During attempts to derive new tissue culture lines of MC-D, foci of rapidly proliferating lymphoblast-like cells (LBC) appeared in some cultures between days 10 and 20; these cells had the capacity to kill all spindle-shaped tumor cells within the ensuing 2 days (Fig. 1). This phenomenon was observed in 8 of 17 primary MC-D cultures, whereas similar changes were absent in 13 primary testis and 8 primary kidney cultures, although some of these normal cell cultures were prepared from tissues of tumor-bearing animals. Tumor cell suspensions prepared by trypsin treatment of solid tumors and stained with Giemsa contained 1.5 to 2 percent small round cells with lymphoid morphology. When LBC did not appear in the primary MC-D cultures within the first 20 days, new tumor cell lines were successfully derived.

Table 1. Cytotoxic effect of lymphoblast-like cells (LBC) on various target cells (TC). The time between culture initiation and addition of LBC was 24 hours in experiments 1 and 2 and 48 hours in the others. Experiments 1 and 5 were performed in triplicate and the others were done in duplicate; MC-D, methylcholanthrene-induced sarcoma D; GP13, strain 13 guinea pig; GPH, Heston strain of outbred guinea pig; B6, C57BL/6J mice.

Origin of TC	LBC per milliliter ($\times 10^{-5}$)	Incubation of LBC with TC (hours)	TC survival (%)
	Experiment 1		
MC-D	1	72	1.6*
GP13 testis	1	72	10.5
GP13 kidney	1	72	26.3
	Experiment 2		
MC-D	1	72	2.9
GP13 testis	1	72	15.5
GP13 kidney	1	72	11.6
B6 testis	1	72	35.3
	Experiment 3		
MC-D	4.4	24	13.8
B6 melanoma	4.4	24	79.6
B6 polyoma	4.4	24	74.7
	Experiment 4		
MC-D	4.4	72	0.0
B6 melanoma	4.4	72	21.0
B6 polyoma	4.4	72	56.2
	Experiment 5		
MC-D	1	48	11.0
GPH testis	1	48	25.9
GPH kidney	1	48	31.7

* Combined statistical analysis of experiments 1 and 2: survival of MC-D compared to testis, $t = 3.5$ ($P = .010$); survival of MC-D compared to kidney, $t = 3.1$ ($P = .014$).

It was repeatedly observed that LBC did not proliferate in the absence of MC-D cells even when fresh medium was supplied, and gradually lost their viability as judged by dye exclusion (0.2 percent trypan blue). However, their proliferative and cytotoxic potentials were rapidly reestablished on addition of MC-D tumor cells to the culture. This finding is considered to indicate that tumor cells, the probable carriers of the antigenic stimulus, were essential for maintaining the activities of LBC. In fact, LBC could be kept in a state of continuous proliferation and cytotoxicity by a steady supply of MC-D cells for more than 6 months.

The cytotoxic effect of these tumor-derived LBC was investigated on various cell lines established from strain 13 and from outbred Heston guinea pigs or C57BL/6J mice. Cultures of target cells were started with 10^5 cells per milliliter on cover slips in Leighton tubes of 1-ml capacity, and LBC (from an actively destroyed primary tumor culture) were added 24 to 48 hours later. The cover slips (10 by 35 mm) were removed at various times of incubation and stained (Wright stain), and the survival rate of target cells was established by counting the cells in ten microscopic fields (longitudinally 3 mm apart) (Table 1). Although produced only in the presence of MC-D cells, LBC were cytotoxic for syngeneic, allogeneic, or xenogeneic target cells. However, the destruction of MC-D cells was more effective and faster than that of other cell types; normal guinea pig cells were also destroyed completely in 3 to 5 days, but mouse cells were never killed out completely from the culture. In general, target cell destruction could be accelerated by increasing the number of LBC in the system. This cytotoxic effect was found to depend upon the number of viable LBC added to the system; supernatants of LBC

cultures or sonicates of LBC had no effect on target cells even after 96 hours of incubation. There was no evidence for lymphotoxin release, since the cell line L 929 (Microbiological Associates, Bethesda, Maryland), known to be very sensitive to this factor (*4*), was not affected by these supernatants.

The LBC which emerged from primary cultures of tumor cells possessed the following characteristics of effector lymphocytes responsible for in vitro cell-mediated immune reactions (*5*). (i) Upon stimulation by MC-D, LBC incorporated tritiated thymidine and uridine, as detected by autoradiography (*6*). (ii) The LBC were strongly pyroninophilic, as revealed by staining with methyl green–pyronine (*7*). (iii) No immunoglobulin was detected either on the surface of LBC or in the medium of MC-D cultures lysed by LBC (*8*) by Coombs-type mixed agglutination, by indirect immunofluorescence, or by the globulin antibody consumption test (*9*). Rabbit antiserums against guinea pig $F(ab')_2$ and against guinea pig immunoglobulin G_2 and the corresponding fluorescein-tagged sheep antibodies to rabbit globulins were used in these tests. (iv) Media from LBC cultures contained a factor (at 1 : 4 dilution) capable of inhibiting the migration of normal syngeneic guinea pig peritoneal cells from capillaries; this factor was not present in supernatants of MC-D cultures (*10*). In the last-mentioned experiment CMEM served as a control; peritoneal cells were harvested from animals that had received 50 ml of sterile light paraffin oil intraperitoneally 3 days earlier.

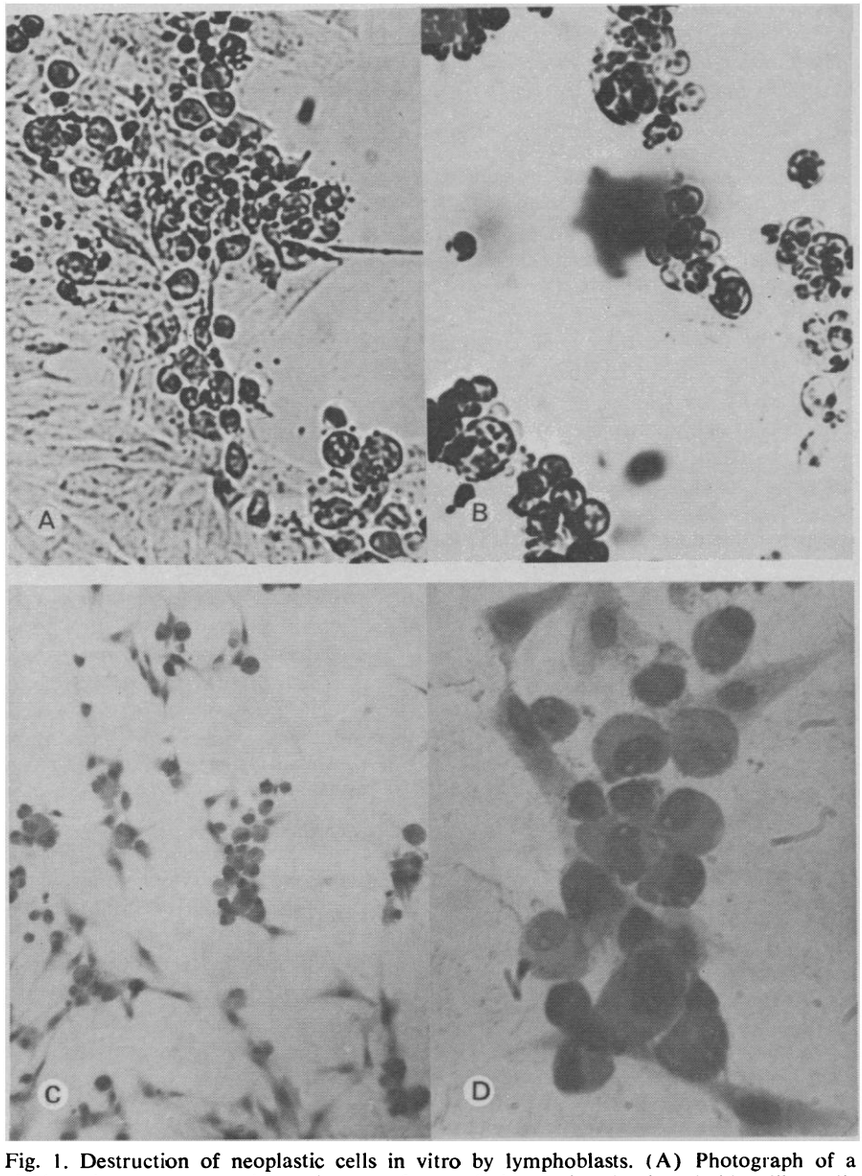

Fig. 1. Destruction of neoplastic cells in vitro by lymphoblasts. (A) Photograph of a viable primary tumor culture shows a focus of destruction by lymphoblast-like cells (LBC), which had emerged 10 days after initiation of the culture. LBC attached themselves to tumor cells and proliferated while lysing the latter (× 400). (B) Complete destruction of tumor cells is seen 2 days later in the same culture. Only one spindle-shaped target cell is visible in the field. Clusters of LBC are floating freely in the medium (× 400). (C) In a cytotoxic experiment with LBC, tumor cells from a tissue culture line were mixed with cytotoxic cells (2.2×10^5 cells of each type per milliliter). The photograph was taken 72 hours later. Only a few spindle-shaped target cells survived, and some of these were in the process of being lysed. (Wright stain, × 170). (D) The culture in C is shown at higher magnification. The LBC have abundant cytoplasm around the eccentric nucleus and large prominent nucleoli (Wright stain, × 600).

The results reported here indicate that, in spite of the inability to demonstrate at this stage strict immunological specificity of cytotoxic action, LBC possessed characteristics of activated thymus-derived lymphocytes. Therefore, it is suggested that this relative lack of specificity represents the potential of immune lymphocytes to kill antigenically unrelated cells after stimulation by specific antigens, as was also invoked in other studies (*11–13*). Alternatively, the apparently broad cytotoxic activity of LBC may be due to antigenic cross-reactivity among different target cells in tissue culture (*14*).

The LBC were capable of destroying tumor cells in vitro, whereas their progenitors, which had infiltrated the tumor and thus had the opportunity of reacting against tumor antigens in vivo, were unsuccessful in bringing about spontaneous regression in this tumor-host system. This striking difference might be due to a tumor-enhancing mechanism or mechanisms operating in vivo which may involve humoral antibodies (*15*); it must be stressed in this context that no immunoglobulin production was detected in this culture system. Besides the possible role of immunological enhancement, the participation of other as yet unknown factors in this phenomenon cannot be excluded at this time.

The appearance of lymphocytes in a primary culture of human neoplastic tissue had been previously reported (*12, 16*); moreover, these lymphocytes

eventually destroyed the tumor cells and were shown to be cytotoxic also to various human tumor cell lines. Similarly, peripheral blood leukocytes from patients with Burkitt's lymphoma responded in vitro to autochthonous tumor cells with increased DNA synthesis and cytotoxicity, but the progenitor leukocytes did not exert cytotoxicity before in vitro stimulation (*17*). The parallelism between these findings and the results reported here would support the view that lymphocytes sensitive to tumor antigens may be present in both animal and human hosts and that, although these lymphocytes may be inefficient in vivo in suppressing tumor growth, they have the potential of mounting a strong cytotoxic reaction after stimulation in vitro.

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29 January 1973

Blood-Feeding Requirements of the Mosquito: Geographical Variation in Aedes taeniorhynchus

Abstract. *Wild populations of* Aedes taeniorhynchus *were polymorphic for the diets required for ovarian development. In* Aedes taeniorhynchus *populations from mangrove swamps, most females possessed the capacity to produce eggs on a blood-free diet. But where the grassy salt marsh was the mosquito's principal habitat, most females lacked this capacity. Both kinds of females could utilize a blood meal for egg production.*

In biting flies such as mosquitoes (Culicidae), blackflies (Simuliidae), sand flies (Ceratopogonidae), and horseflies (Tabanidae), only the females take blood. Males do not have biting or piercing mouthparts and usually feed on plant nectars. The females also feed on plant sugars, but, with numerous exceptions, ovarian development is dependent on a blood meal. Many blackflies of the arctic and subarctic regions have only vestigial biting mouthparts and are nonfeeders as adults (*1*). Some of the ceratopogonids of these regions show similar modifications (*2*). Even when equipped for blood-feeding, some species do not need a blood meal to develop and lay an initial egg batch. This capacity, which is called autogeny, occurs in all four of the families mentioned above (*1*, *3*, *4*).

We report that *Aedes taeniorhynchus* populations from mangrove swamps have a much greater capacity to produce eggs on a blood-free diet than those populations occurring in grassy salt marshes. The North American distribution of this species ranges from the equator to 41°N latitude. The

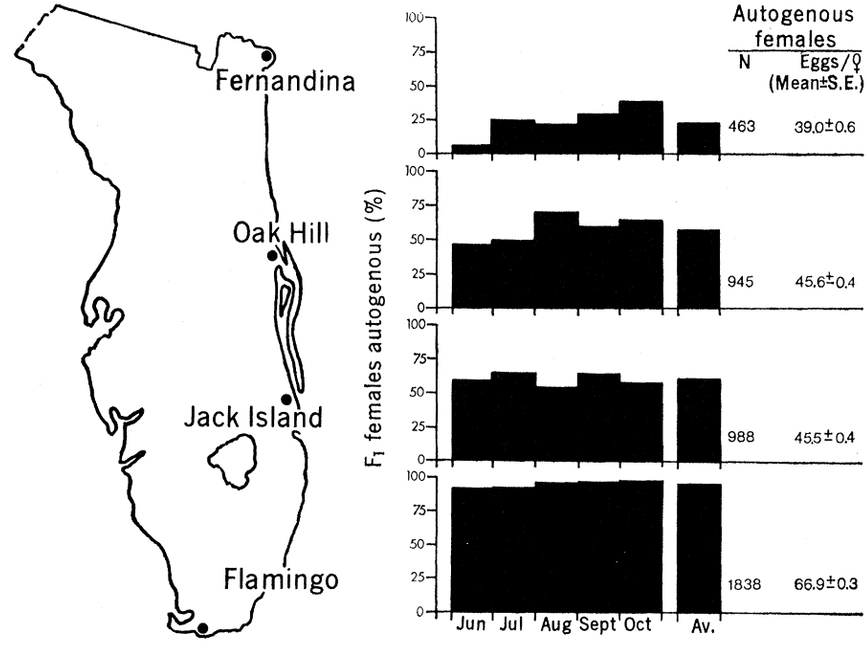

Fig. 1. The frequency and the expression of autogeny in F_1 *A. taeniorhynchus* females derived from biting collections at four coastal sites in Florida. Monthly field collections were made at each site from June through October 1971.