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Inhibition of Tumor-Induced Lymphocyte Blastogenesis by a Factor or Factors Associated with Peripheral Leukocytes

Abstract. *A factor or factors concentrated on leukocytes of cancer patients depress the proliferative response of lymphocytes to stimulation with autologous tumor. Inhibitory activity with material eluted from cells resides in neither high nor lower molecular weight fractions but in a combination of the two. The finding suggests that in vitro inhibition of lymphocyte proliferation to autochthonous tumor occurs because of antigen-antibody complexes.*

A factor or factors which block the proliferative response of lymphocytes to stimulation with autologous tumor can be detected in patients with primary malignancies (*1*). In our experiments inhibitory factors are concentrated on leukocytes and are removed from these cells by elution through washing with a subsequent increase in the cells' proliferative response. Crude fractionation of the material recovered in the eluate suggests that antigen-antibody complexes may be responsible for this blocking.

Many animal and human neoplasms elicit in the primary host a response against the tumor which can be measured by various serological and cell-mediated immune reactions (*2*). One such method is the mixed lymphocyte–tumor cell interaction (MLTI), which is a variant of the mixed lymphocyte culture, but recognizes tumor-specific antigenic differences (*3*). Of the various mechanisms that may effect cellular immunity, serum blocking factors have been extensively studied for their ability to inhibit the cytotoxicity of immune lymphocytes in vitro. Blocking components have been examined in tumor-bearing animals and most likely represent antigen-antibody complexes or soluble tumor antigen whose mechanism of action involves both an interaction with tumor cells and with effector lymphocytes (*1*, *4*).

Tumor specimens and normal tissues from the tumor-containing organ were obtained at the time of operation from patients who had received no other therapy for their neoplasms (seven carcinomas of the lung and one cancer of the stomach). The tissues were mechanically dispersed under sterile conditions after passage through 500- and 140-μm stainless steel sieves. Cellular material was then suspended in culture medium RPMI 1640 and layered on top of Ficoll-Hypaque gradients (*5*) which were centrifuged at 175*g* for 20 minutes (*6*). Cells were washed three to five times in RPMI 1640 at 24°C and then incubated overnight in the same medium at 37°C in a 5 percent CO_2 and air mixture prior to final washing and irradiation with 1500 r. Normal tissue from the cancerous organ was treated in a similar fashion (*7*). Peripheral blood drawn before surgery was separated on Ficoll-Hypaque gradients. Cells recovered at the interphase consisted of more than 90 percent lymphocytes, the remainder being classified as macrophages and granulocytes. Any contaminating red cells were removed by exposure to tris-buffered isotonic ammonium chloride (*8*, *9*). Various ratios of stimulating to responding cells were tried, but most cultures were performed with 2×10^5 responding lymphocytes and 1×10^5 irradiated stimulating cells in 0.2 ml of medium containing either autologous or normal plasma. Cultures were incubated for 3, 5, or 7 days. The harvesting and counting methodology has been described (*10*).

Table 1. Incorporation of tritiated thymidine in peripheral lymphocytes of cancer patients after autologous tumor stimulation, comparing a one-wash (W1) versus a five-wash (W5) procedure. The values are the means of five cultures ± the standard errors of the means.

Patients *	Peripheral lymphocyte responses † (count/min)			
	W1 lymphocytes		W5 lymphocytes	
	+ Tumor	+ Normal tissue ‡	+ Tumor	+ Normal tissue ‡
N.B.	16,869 ± 3,071	931 ± 74	58,721 ± 1,754	1,783 ± 452
H.S.	1,083 ± 97	690 ± 43	3,570 ± 186	404 ± 45
I.R.	3,271 ± 764	1,522 ± 101	15,074 ± 3,004	3,276 ± 200
B.E.	783 ± 53	990 ± 80	3,041 ± 178	597 ± 38
R.A.	2,910 ± 128	1,077 ± 194	5,938 ± 369	576 ± 66
S.S.	4,806 ± 301	1,331 ± 129	10,563 ± 1,243	1,648 ± 137
E.D.	945 ± 86	878 ± 116	2,810 ± 103	935 ± 45
J.B.	6,122 ± 505	1,899 ± 178	13,488 ± 2,061	2,111 ± 206

* Patient N.B. had an adenocarcinoma of the stomach; the others had squamous cell carcinoma of the lung. † Five-day cultures, values for W5 lymphocytes greater than those for W1 lymphocytes at all time intervals, that is, 3 and 7 days in culture. ‡ Normal tissue counterpart after 1500-r irradiation.

Table 2. Inhibition of autologous tumor-induced lymphocyte proliferation by ultrafiltrates from material eluted from a cancer patient's peripheral leukocytes. The patient (J.B.) had squamous cell carcinoma of the lung. Cultures were set with 2×10^5 responding lymphocytes stimulated with 1×10^5 irradiated tumor cells. The values are tritiated thymidine uptake (in counts per minute) for 5-day cultures: mean values for five cultures ± standard errors of the means.

Material tested for blocking activity		Patient J.B. lymphocytes + patient J.B. tumor	
Eluate	Fraction	W1 lymphocytes	W5 lymphocytes
None (control)	Control	2,971 ± 478	11,018 ± 1,062
Eluate from patient J.B. leukocytes	CW *	2,416 ± 300	3,141 ± 294
	CW_{10}	3,603 ± 249	9,003 ± 806
	CW_{100}		10,181 ± 1,817
	CW_{10+100}	2,195 ± 91	2,001 ± 94
Eluate from normal (control) leukocytes	CW *	3,694 ± 600	14,108 ± 549
	CW_{10}		
	CW_{100}		8,996 ± 900
	CW_{10+100}		11,087 ± 989

* Added at equivalent protein concentrations.

In Table 1 the differences in MLTI blastogenic responses for W1 (*8*) versus W5 (*8*) lymphocytes are depicted (*11*). These findings have now been confirmed in over 30 different experiments with fresh or frozen cells. Results are similar whether cultures are performed in autologous or normal plasma. The implication that factors eluted from W1 cells are inhibitory is strengthened by the observation that W5 but not W1 cells are inhibited by the presence of the cell wash eluate in the culture scheme (Table 2). An active inhibitory component in the cell wash (CW) eluate can be fractionated and results in high and low molecular weight fractions (CW_{100} and CW_{10}). When combined, inhibition is comparable to that for the unfractionated (CW) material. Each fraction alone is inactive (Table 2). Material eluted from normal cells is inactive in a similar setting. Inhibition of W5 lymphocytes by the cell wash eluate is reversible. Since cells regain their capacity to respond following a removal of the inhibitory material, a cytotoxic effect can be ruled out.

The eluate has immunological specificity. The W5 cells from lung carcinoma patients do not show inhibition when preincubated with the eluate from the patient with adenocarcinoma of the stomach (*12*).

Furthermore, an inhibitory factor or factors present in the cell wash can be absorbed out with the patient's W5 cells but not with tumor cells. These findings differ from those of other workers where blocking factors in a cytotoxic assay could be specifically removed from serum only after absorption with target cells (*9*). In summary, the data demonstrate that peripheral leukocytes in cancer patients carry an inhibitory factor or factors. The active component resides in neither high nor low molecular weight components but in a combination of the two and suggests antigen-antibody complexes. This material is not cytotoxic but prevents the full expression of lymphocyte proliferative capacity in response to stimulation with tumor cells. Loss of inhibition might be explained on the basis of low affinity binding of an inhibitory factor or factors at the cell surface or the rapid turnover and reconstitution of lymphocyte receptors at the membrane level (*13*).

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6. Cytological examination of the cell suspensions recovered at the bottom of the gradient revealed carcinoma cells of squamous type in various stages of differentiation for the pulmonary neoplasms and for the remaining tumor, a well-differentiated adenocarcinoma of the stomach.
7. The malignant potential of the tumor cells was confirmed by their ability to grow and form histologically similar neoplasms in the thymus-deficient "nude" mice. For future testing, tumor and normal tissue were stored in a frozen state.
8. This suspension of cells underwent one 5-minute wash (W1 cells) in RPMI 1640 at 24°C. After centrifugation at 300*g* for 10 minutes the supernatant was saved and an aliquot of W1 cells was washed three more times, followed by a 12-hour incubation at 37°C in a 5 percent CO_2 and air atmosphere. Lymphocytes recovered at this point (W5 cells) were greater than 80 percent viable by trypan blue exclusion. All supernatants were combined (cell wash eluate) and concentrated tenfold by filtration. A portion of the cell wash eluate (CW) was adjusted to a *p*H of 3.1 in NaCl-glycine buffer and passed through a series of membranes of determined pore size by ultrafiltration. Material retained by the Amicon filter X M100 (CW_{100}) contains molecules above 100,000 daltons, including immunoglobulin. Subsequent passage of the filtrate from the X M100 step through an Amicon PM-10 membrane results in material of 10,000 to 100,000 daltons (CW_{10}). Collected fractions were adjusted to *p*H 7.2 prior to assaying (*8*).
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11. Responses for W5 cells were higher than those for W1 cells at all time intervals in culture.
12. Upon preincubating W5 lymphocytes or tumor cells with the CW eluate the only clear effect is upon the responding lymphocyte and not the stimulating tumor cell, that is, for patient H.S., W5 lymphocytes + CW incubated 1 hour at 24°C, rinsed once and reacted with irradiated tumor gave 1421 count/min ± 265 (S.E.); tumor + CW incubated 1 hour at 24°C, rinsed once, irradiated and reacted with W5 lymphocytes gave 7686 ± 339 (S.E.). The control consisted of W5 lymphocytes from patient H.S. incubated with an eluate from patient N.B., rinsed and reacted with irradiated tumor from H.S. gave 4234 ± 416; W5 lymphocytes + tumor (no eluate) gave 4570 ± 186.
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Pierce's Disease of Grapevines: Evidence for a Bacterial Etiology

Abstract. *A Gram-positive, rod-shaped bacterium was isolated and grown in pure culture on artificial mediums from the leafhopper* Draeculacephala minerva *Ball which had fed on plants infected with Pierce's disease. The bacterial culture was injected into uncontaminated leafhoppers which were than allowed to feed on healthy grapevines. After exposure to these leafhoppers, typical symptoms of Pierce's disease developed; however, no symptoms developed on plants exposed to leafhoppers injected with sterile culture medium. The same organism was reisolated from the experimentally inoculated plants. Electron microscopic examination of these infected plants revealed bacterial cells localized in the xylem tissues. No such cells were seen in healthy or control plants.*

Since 1884, Pierce's disease has been an important transient and insidious problem of grapevines in California and in the southeastern United States. This disease has been investigated intermittently for several decades, yet the etiologic agent remained completely obscure. At first, Pierce (*1*) believed that a microorganism was the causal agent, since bacteria-like organelles were observed in diseased plant material; but he was unable to culture any bacteria that would cause the disease. Weimer (*2*) investigated a very similar disease called alfalfa dwarf. He, too, observed bacteria or bacteria-like bodies in diseased plants but could not isolate any pathogenic bacteria. He showed, however, that the alfalfa dwarf agent was transmitted to healthy plants through grafting, and on this basis he concluded that a virus rather than a bacterium was the cause. Pierce's disease, like alfalfa dwarf, is transmissible by grafting in grapevines and, therefore, it also was concluded to be caused by a virus (*3*). In addition, Hewitt *et al.* (*3*) established that leafhoppers were natural vectors of the disease and later they concluded that Pierce's disease and alfalfa dwarf were incited by the same infectious agent. This agent was shown to be limited to xylem tissues that often became plugged with gum and tyloses