Diffusible Cytotoxic Substances and Cell-Mediated Resistance to Syngeneic Tumors: In vitro Demonstration

Abstract. *The interaction between target cells (mouse sarcoma cells) and syngeneic immunocytes (peritoneal cells) in close proximity but without direct contact was studied. The two cell types were separated by Millipore membranes, which allow diffusion of substances of large molecular size, in an assembly which permits cultivation of target cells on one side of the membrane and immunocytes on the other side. When brought into proximity in this manner for 48 to 72 hours, immunocytes from donors which had been immunized against syngeneic tumors caused destruction of the target cells. Since serum from immunized donors had no effect, it appears that the immunocytes produced a diffusible cytotoxic substance (or substances) which may be different from typical antibody.*

Numerous studies suggest that cell-mediated specific immune reactions, in contrast to serum antibody reactions, are major forces in the rejection of allografts (homografts) of normal or neoplastic tissues (*1*). Experimental data (*2, 3*) suggest similar mechanisms in resistance to syngeneic and autochthonous tumors. However, the mechanism whereby the immunocytes exert their effect, and whether direct contact of immunocyte with target cell is necessary, is unknown. We have designed a method to determine whether lymphoid cells from animals which are immune to syngeneic tumors release cytotoxic substances capable of acting at close range to, but without actual contact with, the target cell.

The use of Millipore membranes to determine whether biological phenomena require direct participation of cells has been reported by several investigators, notably Grobstein (*4*), who studied induction in embryonic tissues in vitro, and Algire and co-workers (*5*), who studied the growth of allogeneic and xenogeneic tissues implanted within Millipore chambers.

An assembly consisting of two compartments separated by a Millipore membrane (*6*) was constructed by cementing the membrane between plastic rings. The target cell compartment consisted of one plastic ring 2 mm deep with an interior diameter of 10 mm. Three 0.1-mm plastic legs were cemented under this ring. Two plastic rings were cemented to the other side of the Millipore membrane, producing an immunocyte compartment 4 mm deep by 10 mm in diameter. Completed assemblies were sterilized by irradiation with 20,000 rad from a ^{60}Co source.

Several fibrosarcomas induced by 20-methylcholanthrene in C57BL/6 female mice and maintained by serial passage in syngeneic mice and in tissue culture less than 12 months were used. Transplantation studies demonstrated the antigenicity of these tumors in syngeneic mice (*7*). Several methods in vitro demonstrated the cytotoxic activity of immunocytes (peritoneal cells or spleen cells) from immunized syngeneic mice and the lack of effect of serum from the same donors when in direct contact with tumor cells (*3*).

We immunized syngeneic mice by injecting living tumor cells subcutaneously, excising the resulting tumors, and then again injecting live tumor cells subcutaneously. About 1 week before the immunocytes were used for experiments, 10^6 tumor cells were injected intraperitoneally. The second and third injections did not produce tumors. Unimmunized controls were untreated syngeneic mice.

Cells from tissue culture lines of these tumors were used as targets. The cells were harvested from monolayer cultures by treatment with trypsin, washed twice with solution A (*8*), and resuspended in tissue culture medium at a concentration of 2.5×10^6 cells per milliliter. Several of the Millipore assemblies were placed in a plastic petri dish 52 mm wide containing 10 ml of tissue culture medium, and 5×10^5 tumor cells (0.2 ml) were pipetted into the target cell compartments. After incubation overnight in a 5 percent CO_2 atmosphere at 37°C to permit attachment of the target cells to the membrane, the assemblies were inverted and returned to the petri dish, and the immunocytes were added.

Peritoneal cells to be used as immunocytes were harvested by lavage of the peritoneal cavity of the donors with 10 ml of solution A. Cells from three to six immune or nonimmune mice were pooled for each experiment. The cells were sedimented by centrifugation at 20*g* for 5 minutes, washed twice with solution A, and resuspended in tissue-culture medium (*9*) at the desired concentration. Five million nucleated cells (0.2 ml) were pipetted into each immunocyte compartment. The petri dish containing these preparations was then incubated for another 48 or 72 hours.

Spleen cells were used as the immunocytes in one study. Spleens from immunized or nonimmunized C57BL/6 mice were minced in solution A. Tissue fragments were allowed to sediment, and the remaining cell suspension was processed as for peritoneal cells. Serum from immunized mice (0.2 ml undiluted serum) was used in two experiments in parallel with studies of peritoneal cells from the same animals.

After incubation, the assemblies were washed gently with isotonic saline, fixed in 95 percent ethanol, and allowed to dry. The membranes were removed and placed on small drops of Harleco synthetic resin mounting medium (*10*) on microscope slides, with the target cell side up, and stained with Giesma. The resin prevented staining of the immunocytes but did not interfere with staining of the target cell monolayer. A cover slip was then affixed.

An experiment usually included studies with immunocytes from immunized donors, nonimmunized donors, and controls with no immunocytes. Because of varying objectives, one or another of these three variables was omitted from some experiments, but since we found that immunocytes from nonimmune mice never affected the tumor cell monolayer when incubated for 72 hours or less, the effect of the immunocytes from immune donors could be evaluated against the preparations with either nonimmune immunocytes or no immunocytes. Evaluation of the effect of immunocytes from nonimmunized donors, or x-irradiated immunocytes, or x-irradiated tumor cells (in the immunocyte compartment) were usually based on comparison with Millipore assemblies containing only the tumor cell monolayer, but any setup which gave heavy growth of the target was accepted as showing no tumor destruction even if the control consisting of tumor cells alone was lacking. In half of the experiments each variable was tested in duplicate, but in others the supply of immunocytes was insufficient for duplicate preparations. To evaluate

Table 1. Number of experiments in which destruction of tumor cell monolayer occurred, as compared to the number of experiments performed. Tabulation includes all experiments with peritoneal cells (PC) as the immunocytes and an incubation time of 48 or 72 hours.

Contents of immunocyte compartment	Target cell line and pore size (μ) of Millipore membrane										
	Tumor VIII			Tumor IX			Tumor XI			Human PK27	Rat Caudoma
	1.2	0.45	0.22	0.01	1.2	0.45	1.2	0.22	0.01	1.2	1.2
Antitumor VIII PC	6/6	5/5	2/2	0/1						0/3	0/3
Antitumor IX PC					1/2	2/2					
Antitumor XI PC							4/5	1/1	1/1		
Nonimmune PC	0/4	0/3	0/2	0/1		0/1	0/5	0/1	1/1	0/3	0/3
Irradiated tumor	0/6	0/3	0/1	0/1			0/1				
Irradiated antitumor PC	0/3	0/1	0/1				0/1		0/1		

the effect of immunocytes on the subjacent target cells, growth of the tumor cell monolayer in each Millipore assembly was graded macroscopically, on the basis of staining intensity, as no growth (0), slight growth (+), moderate growth (++), or heavy growth (+++). Macroscopic evaluation was confirmed by microscopic examination. A decrease in growth from ++ to 0 or + was accepted as significant inhibition.

Incubation for 2 to 3 days was optimum for demonstration of the cytotoxic effect. In one experiment, serial observations were made after incubation for 6, 24, 44, and 48 hours. No change was evident in the target cell monolayer until 44 hours. This rather slow process of target cell inhibition also occurred in studies of cell-mediated immunity in this same tumor system by other techniques (*3*) in which immunocytes and tumor cells were admixed. Incubation for 4 to 7 days proved satisfactory in some experiments, but in others there was deterioration of the target cell monolayers in assemblies containing immunocytes from nonimmunized donors, thus preventing interpretation of specific immune effects in experiments that lasted longer than 72 hours.

When the immunocyte chamber contained peritoneal cells from mice specifically resistant to tumor VIII, the monolayer of tumor VIII cells on the other side of the membrane was strikingly inhibited (Fig. 1). The target cell monolayer grew well when the immunocyte chamber was empty, or when it contained peritoneal cells from nonimmunized syngeneic mice or serum from the immunized or nonimmunized mice. These results indicate that the peritoneal cells from syngeneic mice specifically immunized against tumor VIII yielded a substance (or substances) which destroyed or inhibited tumor cells, and which diffused through membranes that do not permit the transmigration of either target cells or immunocytes. In almost all experiments, the tumor cell monolayer was destroyed when live peritoneal cells from specifically immunized syngeneic mice were incubated in the immunocyte compartment for 48 to 72 hours (Table 1).

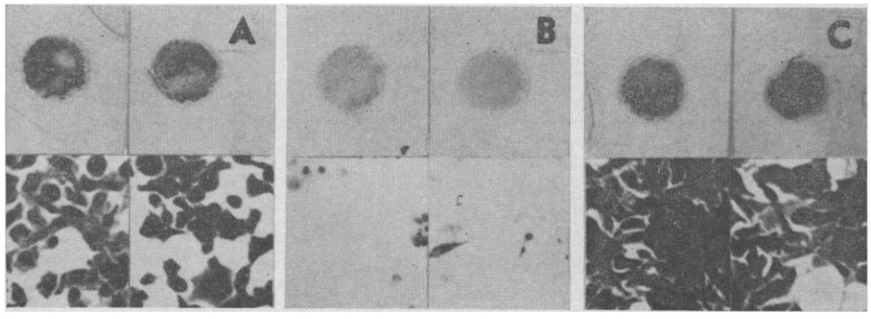

Fig. 1. Millipore membranes, after removal from the assemblies, stained to show tumor cell monolayers only (top), and photomicrographs (× 100) of segments of the stained membranes (bottom). There was good growth of the target cell monolayer on the two membranes from chambers containing no immunocytes (A) and on those from assemblies containing peritoneal cells from unimmunized mice (C), but there was little or no growth of target cells in the assemblies which contained peritoneal cells from specifically immunized donors (B). (Tumor VIII, GS Millipore membranes of pore size 0.22 μ, 48 hours incubation after addition of immunocytes.)

Spleen cells were used as immunocytes in one study. Tumor IX was the target and membranes were of pore size 0.22 μ. Spleen cells from immunized syngeneic donors destroyed the tumor monolayer, but spleen cells from nonimmunized donors did not. A parallel study of peritoneal cells from the same donors gave similar results.

Peritoneal cells from specifically immunized syngeneic donors destroyed target cell monolayers of three methylcholanthrene-induced sarcomas of C57BL/6 female mice (tumors VIII, IX, and XI). In contrast, peritoneal cells from nonimmunized donors destroyed the target cell monolayers in only 1 of 18 studies. Cross-reactions between the three mouse tumors were not studied, but a human cell line (melanoma PK 27) and a rat Caudoma line (*11*) were not destroyed.

As an additional control, tumor cells irradiated by ^{60}Co to 10,000 rad were placed in the immunocyte compartment to provide metabolizing, but damaged and nonpropagating, cells incapable of any immunologic reaction against the target, but which might release toxic products. There was no effect on growth of the tumor cell monolayer.

Irradiation of peritoneal cells from immunized mice (10,000 rad from a ^{60}Co source) abolished their cytotoxic effect on the tumor cell target. Irradiation also prevented the cytolytic effect of immunocytes in cell mixtures (*12*).

The deterioration of target cell monolayers in several experiments after 4 to 7 days' incubation subjacent to nonimmunized peritoneal cells, without corresponding deterioration of target cells in the control preparations consisting of tumor cells alone, suggested the possibility of a response of uncommitted immunocytes to the previously unencountered tumor specific antigen, that is, if such antigens should diffuse through the Millipore membrane into the immunocyte chamber. To test this possibility we mixed 10^5 or 10^6 irradiated tumor cells with 5×10^6 live immunocytes from nonimmunized donors and placed them in the immunocyte compartment. In each of four studies with syngeneic peritoneal cells and in three of eight studies with spleen cells, the target cell monolayer subjacent to these mixtures deteriorated after incubation for 4 to 7 days. In the simultaneous studies without admixed tumor cells in the immunocyte compartment, tumor cell destruction occurred less frequently (in two out of four and two out of eight, respectively).

Thus in three of the experiments the results suggested that direct contact with tumor cell antigens caused a more rapid or more effective sensitization of the immunocytes. However, in similar studies with genetically foreign immunocytes, the tumor target was damaged in only one of three experiments with rat (xenogeneic) immunocytes, and in none of three studies with C3H mouse (allogeneic) cells. In the absence of more consistent results with syngeneic immunocytes, and until the failure of genetically foreign cells to react is explained, we cannot conclude that sensitization of immunocytes in vitro occurred in this system.

Tumor-specific resistance and homograft immunity are mediated by cellular rather than by humoral mechanisms. Numerous studies show that transplantation resistance can be transferred by immunocytes but not by serum. These characteristics apply to the tumor system in our studies (*3*). Our results, which indicate that cell-mediated immunologic resistance to a syngeneic tumor may actually be mediated through a diffusible cell product, rather than by actual contact of immunocytes and target cells, seem contradictory to the concept of a cell-mediated mechanism which is distinct from humoral antibody. Various explanations for the apparent discrepancy can be considered.

Passage of cells through the membrane was not detectable in these or other studies (*4, 5*) with 0.45-, 0.22-, and 0.10-μ membranes. The possibility that direct cell-to-cell contact was accomplished by penetration of pseudopodial cell processes through the pores of the membrane cannot be completely excluded, but it seems unlikely in view of the characteristics of Millipore membranes and the large numbers of immunocytes that are required to destroy these tumor cells in direct mixtures (*3, 4*).

Cytotoxicity due to liberation of typical antibody by the immunocytes can not be completely excluded, even though serum was not cytotoxic. Antibody might be removed from serum in vivo by reaction with antigen or by adsorption onto immunocytes ("cytophilic antibody"), but might accumulate in our experiments because of the artificial conditions. However, such explanations are probably not true, because there is no known source of complement in the experimental system.

The diffusible cytotoxic substance may be a new class of humoral "defense mechanism," differing from typical antibody in that it acts only if immunocyte and target are in very close proximity, undergoing a striking loss of effectiveness with either time or dilution.

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References and Notes

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9. Tissue culture medium was Eagle's minimum essential medium containing glutamine (Microbiological Associates, Bethesda, Md.) with 20 percent "a-gamma" calf serum (Hyland Laboratories, Los Angeles, Calif.) and penicillin (100 I.U./ml) and streptomycin (200 μg/ml).
10. Hartman-Leddon Co., Philadelphia, Pa.
11. Human melanoma cell line PK27 was established in this laboratory and shown to contain malignant melanoma cells by its growth when transplanted into immunologically tolerant rats (K. Kikuchi and C. M. Southam, unpublished). Rat Caudoma line was established in this laboratory by Miss V. I. Babcock from a spontaneous fibrosarcoma of the tail of a Wistar rat and proved to contain malignant sarcoma cells by its growth on homotransplantation into newborn rats.
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7 March 1969

Ion Exchange in Escherichia coli: Potassium-Binding Proteins

Abstract. *The potassium binding that occurs during passive accumulation by intact* Escherichia coli *has been demonstrated in a cell-free system. The data suggest that this binding is due largely to binding by the cell proteins in general and cannot be accounted for by the binding of a particular protein. The results are in accord with the view that the ion-exchange properties of protoplasmic polyelectrolyte determine the overall ionic composition of the cell.*

Unique proteins have recently been implicated in the cellular transport of β-galactoside (*1*), sulfate (*2*), and leucine (*3, 4*), but binding proteins involved in the transport of potassium have not been described.

The recent finding in this laboratory that significant amounts of potassium were taken up by *Escherichia coli* in the absence of an energy source (Fig. 1) and the simplicity of this "passive" uptake suggested that potassium binding

Table 1. Binding by intact cells and subcellular fractions. Cells harvested during the logarithmic phase of growth in medium KA were K-depleted and fractured as described in the text. Fractions derived from bacterial suspensions containing 29 mg dry weight per milliliter were dialyzed in Visking tubing for 3 hours at 20°C against 1 m*M* KCl (*p*H 6.0) or against an aqueous mixture of Na and K salts (3 m*M* KCl–100 m*M* NaCl, *p*H 6.0). The K uptake in these preparations took place in the absence of metabolism. Incubation at 0°C and in the presence of 50 m*M* potassium azide did not alter the result and no acidification of the unbuffered medium occurred during the incubation. The K and Na concentrations are those in the dialysis sacs; the values are the means of three measurements, ± the standard error of the mean.

Row No.	Fraction	Medium		
		1 m*M* KCl	3.0 m*M* KCl–100 m*M* NaCl	
		K concn. (m*M*)	K concn. (m*M*)	Na concn. (m*M*)
1	Medium	1.00 ± .01	3.00 ± .06	100.0 ± 1.0
2	Intact cells	7.08 ± .25	8.30 ± .12	99.0 ± 0.9
3	Fragmented cells	6.48 ± .20	3.90 ± .05	106.0 ± 1.0
4	Supernatant*	6.70 ± .1		
5	Pellet*	(.43 ± .01)†		
6	Supernatant‡	4.35 ± .20		
7	Pellet‡	2.58 ± .03		

* Twenty minutes centrifugation at 17,000*g*. † Potassium concentration of the fragmented suspension due to binding by cell wall and membrane fragments. After equilibrium dialysis, an aliquot of the broken cell suspension was centrifuged at 17,000*g* for 20 minutes. The pellet was resuspended in the same volume of H_2O and the potassium concentration was determined by flame photometry. ‡ Two hours centrifugation at 100,000*g*.