NATURE OF LYMPHOCYTE-TUMOR INTERACTION

A General Method for Cellular Immunoabsorption*

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The phenomenon of lymphocyte-mediated cytolysis in vitro has been demonstrated repeatedly (references 1 and 2 for review), and there is evidence that the same sensitized lymphoid cell population responsible for tumor cell destruction in vitro also functions in tumor allograft rejection in vivo (3). A multistage process that initially involves lymphocyte-target cell binding and ultimately leads to target cell destruction has been demonstrated for lymphocyte-mediated cytolysis (4). Using a new technique for cellular immunoabsorption, we have now investigated the nature of the initial lymphocyte-tumor interaction.

Materials and Methods

Tumors, Animals, and Immunizations.—Leukemia EL4 of C57BL/6 (Jackson Laboratory, Bar Harbor, Maine) as well as mastocytoma P815 and leukemia L1210 of DBA/2 mice (Roswell Park Memorial Institute, West Seneca, N. Y.) were carried as ascites tumors (3, 5). 3–6-mo old BALB/c males (Roswell Park Memorial Institute) were immunized against EL4 by a single intraperitoneal injection of 25×10^6 tumor cells suspended in phosphate-buffered saline (PBS).[1]

Peritoneal Exudate Cells.—BALB/c anti-EL4 crude peritoneal exudate cells were harvested 11 days after immunization, and purified peritoneal exudate lymphoid cells (PEC) were obtained by passage through nylon wool as previously described (6).

Monolayers for Immunoabsorption.—1 ml of poly-L-lysine ([PLL], mol wt 80,000, 50 μg/ml in PBS) (Sigma Chemical Co., St. Louis, Mo.) was added to each 35 × 10 mm polystyrene tissue culture plate (Falcon Plastics, Oxnard, Calif.). After 60 min, plates were washed three times in PBS to remove unbound PLL, and 1 ml of a 1–5% (vol/vol) suspension of tumor cells washed three times in PBS was added. After 30 min, the plates were gently dipped several times in a beaker of PBS to remove unattached cells, and the tumor cell monolayer was covered with fresh PBS for later use.

The PLL method has proved to be highly efficient in the formation of confluent cell mono-

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[1] *Abbreviations used in this paper:* [^{51}Cr]EL4, [^{51}Cr]Na_2CrO_4-labeled EL4 tumor cells; HRBC, human red blood cells; PBS, phosphate-buffered saline; PBS-EDTA, PBS-FCS containing sodium ethylenediaminetetraacetic acid 5 mM, pH 7.2; PBS-FCS, PBS supplemented with 10% heat-inactivated fetal calf serum; PBS-OVA, PBS supplemented with twice-crystallized ovalbumin, 2 mg/ml (Worthington Biochemical Corp., Freehold, N.J.); PEC, purified peritoneal exudate lymphoid cells; PLL, poly-L-lysine.

layers. If the plates were not treated with PLL, only patchy adherence of cells was obtained. Monolayers could be established on PLL-treated plates only in the absence of serum. The stability of monolayers was found to be dependent upon cell type. Microscopic observation revealed firm attachment of 99% of EL4 cells to the surface of the culture plate and viability was 95% as judged by trypan blue staining. Storage of EL4 monolayers for 2–3 h at 7°C did not result in detectable detachment or loss of viability. Mastocytoma P815 and leukemia L1210 cells formed monolayers that were as stable as those formed with leukemia EL4, while red blood cell monolayers were even more stable, in line with the results of Kennedy and Axelrad (7).

Immunoabsorption and Elution of PEC from PLL-Fixed Monolayers.—PBS was decanted from monolayers, and purified PEC (5.0×10^5 cells per plate in 0.5 ml of PBS supplemented with 10% heat-inactivated fetal calf serum, [PBS-FCS]) were immediately added. After gentle agitation, plates were incubated at 37°C for 20 min, rocked (1 cycle/min) on a rocker platform (Bellco Glass, Inc., Vineland, N. J.) for 5 min, and again incubated without agitation for 20 min. Medium was removed by careful pipetting after a 10 min rock (5 cycles/min, 37°C). 1 ml of PBS-FCS was added, the plates were briefly agitated manually, and the medium was removed. These two wash solutions containing "nonadherent" cells were combined. For elution, 1 ml of PBS-FCS containing EDTA 5 mM (PBS-EDTA) was then added, the plates were rocked (5 cycles/min) for 10 min at 37°C, and the medium was removed by pipette. An additional 0.5 ml of PBS-EDTA was added, the plates were briefly agitated manually, and the medium was removed. These two wash solutions containing "adherent" cells were combined. Nonadherent and adherent cells were washed once in excess PBS-FCS before determination of cytolytic reactivity.

In Vitro Assessment of Tumor Cell Lysis.—PEC recovered from two monolayers were mixed with 1.75×10^5 [^{51}Cr]Na_2CrO_4-labeled EL4 cells ([^{51}Cr]EL4) in a total volume of 3.5 ml of PBS-FCS. Three 1-ml aliquots of this mixture were incubated for 1 h and the amount of ^{51}Cr released into the medium was measured as previously described (6). Results are expressed as percent of total release obtained from thrice freeze-thawed samples. Vertical bars in figures indicate the range of triplicate assays. Background release of ^{51}Cr from labeled tumor cells incubated in the absence of PEC did not exceed 8% in any experiment.

RESULTS

Fig. 1 shows the effect of EDTA on the release of ^{51}Cr from labeled EL4 cells by anti-EL4 PEC. At concentrations of 1.0 mM and below, there was no inhibition of ^{51}Cr release, while concentrations of 5.0 mM and above showed maximum inhibition, in good agreement with previously reported data (8). Cell-mediated cytolysis was also found to be dependent upon pH, being maximal at neutral pH and not measurable below pH 5.5 (Fig. 2).[2] Medium more acidic than pH 5.0 was toxic for PEC and EL4 tumor cells. We have previously shown that serum in the reaction medium is required for cell-mediated cytolysis (6) and that temperature influences the cytolytic reaction (4). Since a multistage process has been proposed for cell-mediated cytolysis, it was of interest to investigate the dependence of initial lymphocyte-tumor cell binding upon these factors known to affect cytolysis. We therefore utilized monolayers formed by the at-

[2] ^{51}Cr release in PBS-FCS at pH greater than 8 was found to be an unreliable indication of cell viability because a portion of released ^{51}Cr precipitated and was not separable from intact cells by centrifugation. No precipitation of released ^{51}Cr was detected at pH 4–8.

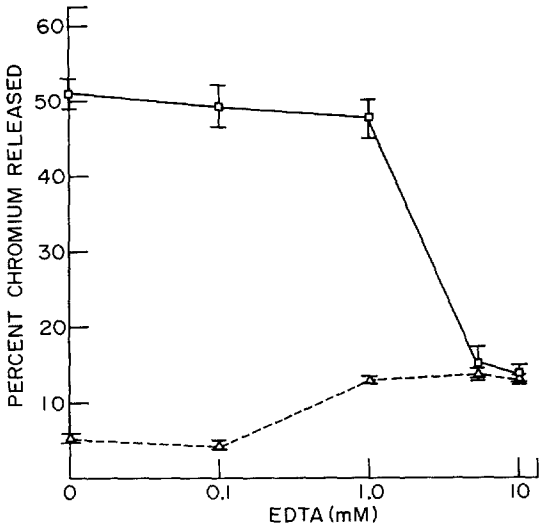

FIG. 1. EDTA inhibition of cytolysis. Cultures containing anti-EL4 PEC (1.5×10^5 cells/plate) plus [^{51}Cr]EL4 (0.5×10^5 cells/plate) (□—□) or cultures containing [^{51}Cr]EL4 alone (△----△) were rocked (5 cycles/min) at 37°C. Released radioactivity was measured after 1 h.

tachment of tumor cells to polystyrene plates with PLL to investigate the initial lymphocyte-tumor cell interaction.

In order to establish the suitability of PLL-fixed monolayers for immunoabsorption, anti-EL4 PEC suspended in PBS-FCS were incubated on various monolayers, and the number, as well as the cytolytic reactivity of nonadherent and adherent cells, was determined (Table I). After the removal of nonadherent cells by PBS-FCS wash (first wash), monolayers were washed a second time with either PBS-FCS or PBS-EDTA, 5 mM (second wash). Cells removed by first and second wash solutions were centrifuged, resuspended in PBS-FCS, and counted. In a separate experiment, recovered cells were tested for their ability to lyse [^{51}Cr]EL4 target cells in vitro. The successful elution with EDTA of effector lymphocytes specifically adsorbed onto EL4 monolayers demonstrated by cell count and cytoytic reactivity confirms that PLL-fixed tumor cell monolayers can function as effective immunoabsorbents and that EDTA can be used for elution of adsorbed cells from the monolayers. Similar specific absorption was seen in five additional experiments, one including L1210 control monolayers (Table II).

Since a component in normal serum is required for cell-mediated cytotoxicity, the role of serum in lymphocyte-tumor cell binding was investigated. Interestingly, absorption of anti-EL4 PEC by EL4 monolayers occurred to a similar extent in the presence or absence of serum (Fig. 3). Although serum is required for some subsequent process leading to target cell lysis (6), the finding that ab-

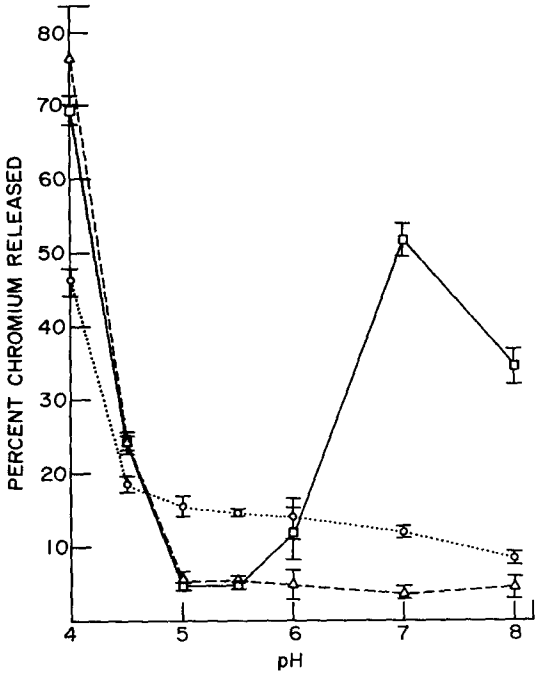

FIG. 2. Inhibition of cytolysis at low pH. Cultures containing anti-EL4 PEC (1.5×10^5 cells/plate) plus [^{51}Cr]EL4 (0.5×10^5 cells/plate) (□—□), ^{51}Cr-labeled PEC (○·····○) alone, or [^{51}Cr]EL4 alone (△----△) in PBS-FCS at pH 4–8 were rocked (5 cycles/min) at 37°C for 1 h.

TABLE I

Immunoabsorption of PEC by PLL-Fixed Cell Monolayers

Monolayer for absorption	First wash				Second wash			
	Solution	Cells eluted*		Lysis‡	Solution	Cells eluted*		Lysis‡
		With PEC	Without PEC			With PEC	Without PEC	
		× 10^{-4}	× 10^{-4}	%		× 10^{-4}	× 10^{-4}	%
None	PBS-FCS	158 ± 3	0 ± 0	65 ± 7	PBS-FCS	1 ± 1	N.D.	6 ± 3
EL4	"	119 ± 1	N.D.§	26 ± 3	"	22 ± 4	13 ± 1	16 ± 2
P815	"	136 ± 4	24 ± 2	41 ± 7	"	16 ± 1	6 ± 3	13 ± 1
HRBC	"	172 ± 2	1 ± 1	65 ± 4	"	0 ± 1	0 ± 1	5 ± 1
None	"	139 ± 4	N.D.	63 ± 3	PBS-EDTA‖	2 ± 1	N.D.	7 ± 1
EL4	"	120 ± 2	28 ± 1	28 ± 3	"	62 ± 7	3 ± 3	66 ± 8
P815	"	138 ± 8	26 ± 3	41 ± 4	"	26 ± 5	6 ± 3	25 ± 5
HRBC	"	184 ± 5	11 ± 1	65 ± 4	"	2 ± 1	0 ± 1	8 ± 4

* Anti-EL4 PEC (1.0×10^6 cells in each of two plates) were absorbed by empty plates or by EL4, P815, or HRBC monolayers (with PEC); PBS-FCS alone was added to control monolayers (without PEC). The number of cells eluted is given as mean ± range of duplicate determinations. Cells eluted with EDTA from EL4 and P815 monolayers differ significantly by Student's t test ($P < 0.05$).

‡ Anti-EL4 PEC (0.5×10^6 cells in each of two plates) were tested for cytolytic reactivity against [^{51}Cr]EL4 after recovery from the indicated monolayers. Data is given as mean ± range of triplicate determinations. Lysis by cells eluted with EDTA from EL4 and P815 monolayers differ significantly ($P < 0.01$).

§ Not determined.

‖ EDTA 5 mM in PBS-FCS, pH 7.2.

sorption can occur in the absence of serum clearly demonstrates that serum factors are not necessary for initial lymphocyte-tumor cell binding.

EDTA inhibition of cytolysis (Fig. 1 and reference 8) and the demonstration of EDTA-mediated elution of specifically absorbed immune PEC from EL4 monolayers (Tables I and II) led to an investigation of the possibility that divalent cations participate in specific PEC-tumor cell binding. Anti-EL4 PEC

TABLE II

Immunoabsorption of PEC by PLL-Fixed Cell Monolayers

Monolayer for absorption	First wash		Second wash	
	Solution	Lysis*	Solution	Lysis*
		%		%
None	PBS-FCS	54 ± 2	PBS-FCS	15 ± 2
EL4	"	27 ± 1	"	17 ± 1
L1210	"	55 ± 7	"	18 ± 1
HRBC	"	54 ± 3	"	10 ± 2
None	"	49 ± 4	PBS-EDTA	23 ± 2
EL4	"	25 ± 2	"	58 ± 4
L1210	"	50 ± 2	"	31 ± 1
HRBC	"	52 ± 4	"	13 ± 1

* Anti-EL4 PEC (0.5×10^6 cells in each of two plates) were incubated on various monolayers. Lysis of [^{51}Cr]EL4 by recovered cells is given as mean ± range of triplicate determinations. Lysis by cells eluted with EDTA from EL4 and L1210 monolayers differed significantly by Student's t test ($P < 0.001$).

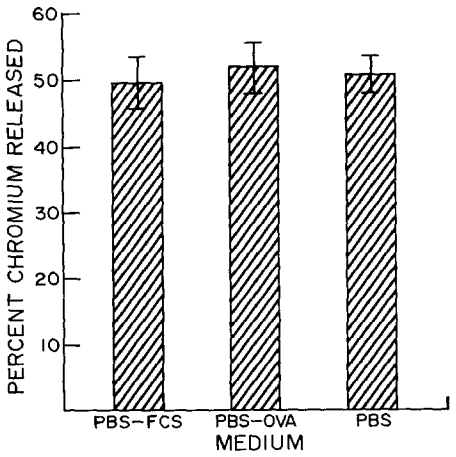

FIG. 3. Effect of serum on immunoabsorption. Anti-EL4 PEC were incubated on EL4 monolayers in PBS-FCS, PBS-OVA, or PBS. Nonadherent cells were removed by PBS-FCS. Adherent cells recovered by PBS-EDTA were tested for cytolytic reactivity against [^{51}Cr]EL4. (Differences are not statistically significant.)

suspended in PBS-ovalbumin (OVA) solutions containing different concentrations of magnesium and calcium were incubated on EL4 monolayers formed by using Ca-Mg-free PBS.[3] Nonadherent cells removed by PBS-FCS wash and adherent cells recovered by subsequent EDTA elution were resuspended in PBS-FCS and tested for cytolytic reactivity against EL4 (Table III). No absorption was evident in the absence of both magnesium and calcium. Partial absorption occurred in the presence of 0.9 and 1.8 mM calcium, but as little as 0.5 mM magnesium was sufficient to produce a striking effect. The absorption seen in the presence of magnesium alone at either concentration tested was not enhanced by the addition of calcium. Magnesium or calcium is, therefore, required for lymphocyte-target cell binding, magnesium being more effective

TABLE III

Effect of Calcium and Magnesium on Immunoabsorption of PEC

Absorption medium*			Lysis by PEC absorbed on EL4 monolayers		Lysis by PEC absorbed on P815 monolayers	
Ca	Mg	Protein	Nonadherent	Adherent	Nonadherent	Adherent
mM	*mM*		%	%	%	%
0.9	0.5	FCS	21 ± 1	47 ± 5	37 ± 3	10 ± 1
0	0	OVA	47 ± 7	8 ± 1	43 ± 2	10 ± 1
0.9	0	"	32 ± 3	23 ± 1	N.D.	N.D.
0	0.5	"	19 ± 1	45 ± 2	46 ± 2	14 ± 1
0.9	0.5	"	20 ± 1	47 ± 2	N.D.	N.D.
1.8	0	"	37 ± 4	32 ± 6	N.D.	N.D.
0	1.0	"	18 ± 1	55 ± 5	45 ± 5	14 ± 4
1.8	1.0	"	16 ± 2	24 ± 2	N.D.	N.D.

* PBS containing calcium and/or magnesium ($CaCl_2$, $MgCl_2$) supplemented by FCS or OVA.

than calcium in mediating such interaction. As can be seen, P815 monolayers failed to absorb anti-EL4 PEC in all of the media tested.

Because deviation from neutral pH was shown to interfere with the cytolytic reaction (Fig. 2), experiments were performed to determine whether adherent cells could be eluted from monolayers at pH values known not to support cytolysis. PEC were adsorbed onto EL4 monolayers and nonadherent cells removed by PBS-FCS wash. PBS-FCS solutions adjusted to pH values between 4 and 7 were then used in a second wash. Cells recovered in the second wash were resuspended in PBS-FCS and tested for cytolytic reactivity (Fig. 4). Elution of adherent cells was, in fact, obtained, demonstrating that lymphocyte-target cell binding can be reversed at low pH and suggesting that binding may involve pH-dependent functional groups on the lymphocyte and/or target cell.

Although cell-mediated cytolysis is not measurable at low temperature, it

[3] OVA was included in the absorption medium to maintain PEC viability and to minimize nonspecific absorption of PEC by any plate surfaces not covered by EL4.

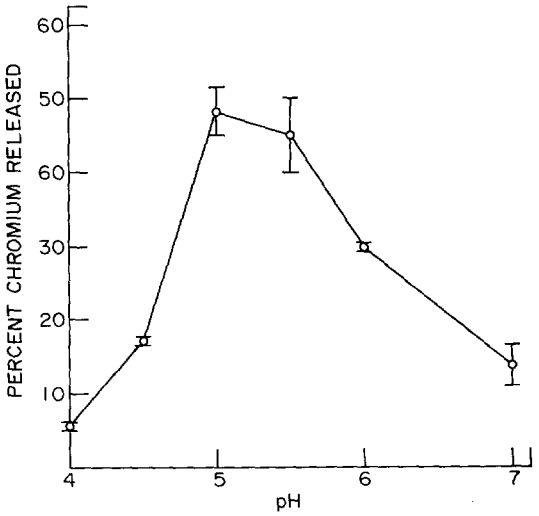

FIG. 4. Recovery of adherent cells at low pH. Anti-EL4 PEC were absorbed by EL4 monolayers and nonadherent cells were removed by PBS-FCS wash. Cells recovered by a second wash with PBS-FCS at low pH were then assayed for cytolytic reactivity against [^{51}Cr]EL4.

has been reported that the initial phase of the lymphocyte-target cell interaction is only weakly temperature dependent (4). We investigated the temperature dependence of the absorption phenomenon and found that the rate of absorption at 25°C is only slightly less than at 37°C. At 7°C minimal absorption of anti-EL4 PEC by EL4 monolayers occurred consistently in three experiments, but its rate was markedly less than that measured at 37°C. Furthermore, a similar effect was seen at 7°C when P815 monolayers were used, indicating that absorption may not be specific at 7°C (Fig. 5). When EL4 monolayers upon which anti-EL4 PEC had previously been adsorbed were cooled to 7°C, detachment of adsorbed cells occurred (Fig. 6). These results demonstrate that a temperature-dependent process is involved in both the formation and maintenance of specific lymphocyte-tumor cell bonds.

DISCUSSION

We have investigated the initial effector lymphocyte-tumor cell interaction by the use of PLL-fixed tumor cell monolayers. The results demonstrate that the presence of magnesium is an important prerequisite for immune lymphocyte-tumor cell binding leading to tumor cell lysis in vitro. The binding process is a temperature-dependent phenomenon that can be rapidly reversed by EDTA or low pH. These investigations have established a new technique for the immobilization of virtually any cell type that allows immunoabsorption and elution of lymphocytes sensitized against transplantation or tumor antigens.

Lymphocyte-mediated cytolytic reactivity has been presumed to be initiated by the recognition of target cell surface antigen through a specific receptor on the effector lymphoid cell. Nevertheless, no immunoglobulin synthesis by effector lymphocytes has been detected (9). In addition, experiments aimed at

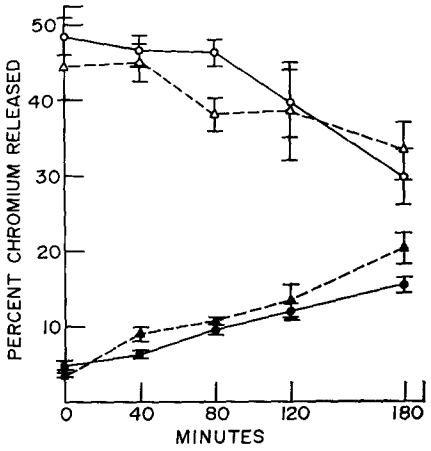

FIG. 5. Absorption of PEC at 7°C. Anti-EL4 PEC were incubated at 7°C for the indicated times on EL4 and P815 monolayers. Adherent cells from EL4 (●—●) and P815 (▲---▲) monolayers as well as nonadherent cells from EL4 (○—○) and P815 (△---△) monolayers were assayed for cytolytic reactivity against [^{51}Cr]EL4.

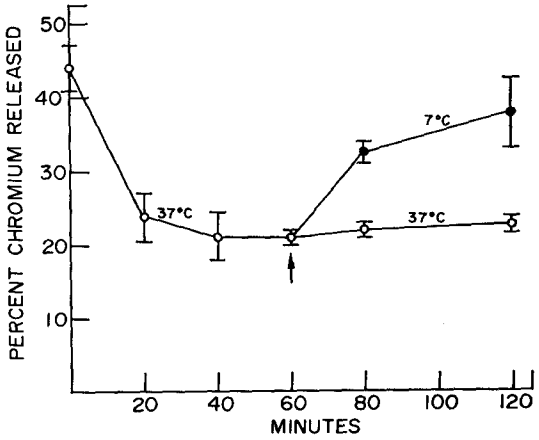

FIG. 6. Elution of adherent cells at low temperature. Cultures containing anti-EL4 PEC and EL4 monolayers were incubated at 37°C. After 60 min (↑) one group of cultures was cooled to 7°C while the other group remained at 37°C. All cultures received gentle manual agitation every 20 min throughout the experiment. Nonadherent cells were recovered at the indicated times and assayed for cytolytic reactivity against [^{51}Cr]EL4. Significant elution of cells ($P < 0.01$) as early as 20 min after cooling was seen in two experiments.

demonstrating immunoglobulin-like molecules on the surface of thymus-derived lymphoid cells, known to be mediators of cellular immunity in general and in vitro cytolysis in particular (10), have so far been inconclusive (11–14). Regardless of the nature of the receptor molecule that plays a role in lymphocyte-target cell binding, possible mechanisms of magnesium (or calcium) participation in this process must be considered. For example, divalent cations could be involved in "bridging" between negatively charged groups on effector lymphocyte and target cell in a ion-triplet bond as discussed by Pethica (15). Alternatively, participation may be more indirect as in the calcium-dependent sheep anti-GAT system (16) where calcium influences the conformation of antigen. It is also conceivable that recognition and binding are discrete events separated temporally or occurring at different points on the lymphocyte-target cell interface during binding. For example, magnesium-independent weak binding of specific receptor and target cell surface antigen may induce changes on the surface of the effector lymphocyte resulting in the activation of stronger, nonspecific, magnesium-dependent binding sites. Since both magnesium and calcium play an important role in cell membrane function, however, the effect of their absence upon lymphocyte-target cell binding may be due to reversible modifications of effector cell membrane processes.

Inhibition of the cytolytic reaction by mildly acidic conditions can be explained on the basis of interference with binding demonstrated by the reversal of absorption at low pH. This pH dependence of the binding process is suggestive evidence for the involvement of cell surface ionic groups whose charge and/or conformation is dependent upon pH. Cell viability was maintained at pH values producing maximal elution; nevertheless, reversible sublethal effects on membrane function or metabolism cannot be ruled out.

While cytolysis is not measurable at 25°C, there is good evidence that the lymphocyte-target interaction can be initiated at low temperature. It has been proposed, therefore, that the cytolytic reaction is a multistage process initiated by lymphocyte-tumor cell binding (4). The finding that absorption occurs at 25°C supports this hypothesis. Specific lymphocyte-tumor cell binding is not entirely temperature independent, since absorption is prevented and even reversed at 7°C. It has also been shown that serum factors are required for the completion of the cytolytic reaction (6). The demonstration that absorption can occur in the absence of serum provides additional evidence for the proposed multistage cytolytic pathway.

Specific absorption of lymphoid cells involved in cell-mediated immunity has been accomplished with macrophage and fibroblast monolayers (3, 17–21) and absorbed cells recovered by trypsinization (3, 18). The recovery of effector lymphocytes from cultured fibroblasts by this method results in abolishment of cytolytic reactivity that persists for a period of time after removal of the enzyme (8). Furthermore, trypsinization causes detachment of fibroblast monolayers and necessitates subsequent separation of absorbed and absorbing cell types (3, 18). In contrast, EDTA effects on cytotoxic lymphocytes are readily

reversible, and PLL-fixed monolayers are bound to the tissue culture plate by electrostatic forces that are not interrupted by EDTA. The rapid selective recovery of absorbed lymphocytes from PLL-fixed monolayers with EDTA is therefore possible.

Cells bound to tissue culture plates by the PLL method showed no detectable change in antigenicity. In addition to the fact that sensitized PEC absorbed specifically onto EL4 cells forming such monolayers, EL4 cells in monolayers were lysed by both anti-EL4 PEC and by anti-EL4 serum in the presence of complement.

Thus far, cellular immunoabsorbents have been limited to those cell types forming adherent monolayers in tissue culture. The formation of immunoabsorbents by electrostatic binding of cells to PLL-treated polystyrene surfaces allows immunoabsorption of sensitized lymphocytes with virtually any cell type. For example, the PLL technique developed here may prove useful for monolayer formation with normal and neoplastic human tissue and would offer an approach to the enrichment and depletion of specific immunologic reactivity in human lymphoid cell populations.

SUMMARY

The binding of sensitized lymphocytes to tumor cells that leads to tumor cell lysis in vitro has been investigated using poly-L-lysine-fixed tumor cell monolayers and lymphocytes obtained from the anatomical site of tumor allograft rejection. The results show that magnesium is an important prerequisite for this interaction and that the extent of lymphocyte-tumor cell binding depends upon temperature as well as pH. Binding can occur in the absence of serum, although serum factors are necessary for the completion of the cytolytic process. The poly-L-lysine technique is applicable to the formation of confluent monolayers with virtually any normal or neoplastic cell type, including those that are otherwise nonadherent to surfaces. Cells immobilized by this technique can be used for the specific immunoabsorption and subsequent recovery of effector lymphocytes sensitized against transplantation or tumor cell antigens.

Note Added in Proof.—Since the submission of this manuscript, it has come to our attention that Golstein et al. (1972. *Eur. J. Immunol.* **2**:380) have reported on immunoabsorption with glutaraldehyde-treated fibroblast monolayers.

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