CELLS MEDIATING SPECIFIC IN VITRO CYTOTOXICITY

I. Detection of Receptor-Bearing Lymphocytes*

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Lymphocytes of mice immunized with allogeneic cells exert a specific cytotoxic effect on these allogeneic cells in vitro (1, and ref. 2 for review). This in vitro effect is thought to reflect similar events in vivo when lymphocytes immune to grafted allogeneic cells, cancerous cells, or even syngeneic cells in autoimmune diseases may destroy these cells. In spite of extensive investigation during the last 10 yr, very little is known about how immune cells kill in vitro. Any explanation would have to account for the specificity of such killing and for the killing itself. Amos (3) has suggested a two-step mechanism: first, specific recognition and then killing by a nonspecific process. Specific recognition would be most simply realized by receptors, on the surface of lymphocytes, complementary to determinants on the surface of target cells.

Most arguments concerning the existence of such specific receptors are based on the observation that immune lymphocytes tend to be adsorbed in greater number on the relevant target cells (i.e., on target cells bearing determinants against which the lymphocytes were immunized) than on nonrelevant ones (4–6). However, these observations do not prove that cells actually involved in killing are preferentially adsorbed. A more complete study has been made by Brondz (7) and Brondz and Goldberg Natali (8), who have incubated immune lymphocytes on monolayers of the relevant target cells for several hours and then washed off the nonadsorbed cells. Two effects were observed: first, the target cell monolayers were destroyed; second, the washed off cells, when put on new target cells, showed low cytotoxic activity. These data were taken as indicating specific adsorption of cells involved in killing, thus proving the existence of specific receptors at their surface. However, the same data could be given other less straightforward interpretations. For instance, instead of being specifically adsorbed on relevant monolayers and thus absent from the washed-off cell population tested for cytotoxicity, cells involved in killing might be present in that population but specifically inactivated by soluble products released by the relevant monolayers; this inactivation hypothesis does not necessarily imply the presence of

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specific surface receptors. A definite proof of specific adsorption, and thus of the existence of specific surface receptors, would be given by elution of the adsorbed cells and demonstration of their killing capacity.

The adsorption-elution experiments described in this paper strongly suggest that there are specific receptors at the surface of cells involved in killing. Moreover, data obtained with cells from animals immunized with two different types of allogeneic cells suggest that these specific receptors are synthetized by the cells that bear them.

Materials and Methods

Mice.—1–2-month old mice from various inbred strains are utilized. We have not detected any effect linked to non-H–2 antigens in this system, in accordance with results obtained by Brondz (9). Thus, a simplified nomenclature will be used throughout this paper for the following strains: A/Sn (*H-2*^{a}, further quoted as a), C57BL/6 (*H-2*^{b}, further quoted as b), BALB/c and DBA/2 (*H-*^{d}, further quoted as d), CBA and C3H (*H-2*^{k}, further quoted as k).

Tissue Culture Medium.—Medium F 13 and Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated (at 56°C for 30 min) fetal calf serum (Bio-Cult, Glasgow, Scotland) with penicillin (75 units/ml) and streptomycin (50 μg/ml) are used throughout the experiments as diluents and tissue culture media.

General Design of Experiments.—A typical core experiment can be described as follows (Fig. 1). K anti-d immune cells are incubated on k or d cell monolayers. At the end of this incubation period, flasks containing the monolayers are shaken; "nonadsorbed" cell suspensions are poured off and tested for cytotoxic activity against d target cells. In some experiments adsorbed cells are eluted by trypsinization and also tested for cytotoxicity; additional information is obtained by density gradient fractionation of these "trypsin-eluted" cells.

Immune Cells.—Mice receive a first intraperitoneal injection of 10^6 immunizing cells and 2 wk later a second intraperitoneal injection of 3×10^7 immunizing cells. These are ascites tumor cells, either from P815-X2, a methylcholanthrene-induced d mastocytoma (10), or from YBB, a Moloney virus-induced k lymphoma. 9–15 days after the second injection the mice are killed, their spleens are minced with scissors and squeezed through a nylon mesh, the resulting cell suspensions are washed once, and adjusted to the desired cell concentration. Lymphoid and nonlymphoid cells are present in these spleen cell suspensions. Although it is generally believed that lymphocytes are the effector cells in specific cell-mediated cytotoxicity, some authors have claimed that nonlymphoid cells may be involved (2). A major involvement of nonlymphoid cells in our system is difficult to reconcile with some of the results described below. Furthermore, experiments to be described in the second paper of this series[1] provide strong arguments for thymus-derived lymphocytes (T cells)[2] to be the only killing cells in this system. Hence, in this paper, we feel justified in referring to nucleated spleen cells involved in killing as lymphocytes or lymphoid cells.

Incubation on Monolayers.—Cell monolayers are prepared in small plastic flasks (25-cm^2 Falcon tissue culture flask, [Falcon Plastics, Oxnard, Calif.]). Macrophage monolayers were

[1] Golstein, P., H. Wigzell, H. Blomgren, and E. A. J. Svedmyr. Cells mediating specific in vitro cytotoxicity. II. Probable thymic origin of receptor-bearing lymphocytes. Manuscript in preparation.

[2] *Abbreviations used in paper*: B cells, nonthymus-derived lymphocytes; BSA, bovine serum albumin; BSS, balanced salt solution; RI, release index; T cells, thymus-derived lymphocytes.

used in preliminary experiments, according to Brondz (7). However, it was soon found that fibroblast monolayers are at least as efficient. For the experiments described in this paper, we use secondary cultures of mouse embryo fibroblasts grown to confluence. 3 ml of immune cell suspension are incubated on each monolayer, at 37°C for 3–4 hr unless otherwise stated, and usually at a concentration of 3×10^6 trypan blue excluding white cells/ml. At the end of the incubation period, each flask is subjected to standardized mechanical agitation (on a flask shaker, Griffin and George, Wembley, Middlesex, England. See Results). The cell suspension is poured off and tested for cytotoxic activity.

Trypsinization Procedure.—After the cell suspension is poured off, each flask receives 5 ml of a 0.25% solution of trypsin in calcium and magnesium-free buffer (Statens Bakteriologiska Laboratorium, Stockholm, Sweden). After a 30 min incubation at 37°C, each flask is shaken, the suspension of trypsin-eluted cells is washed once, is resuspended in 3 ml of tissue culture medium, and is tested for cytotoxic activity.

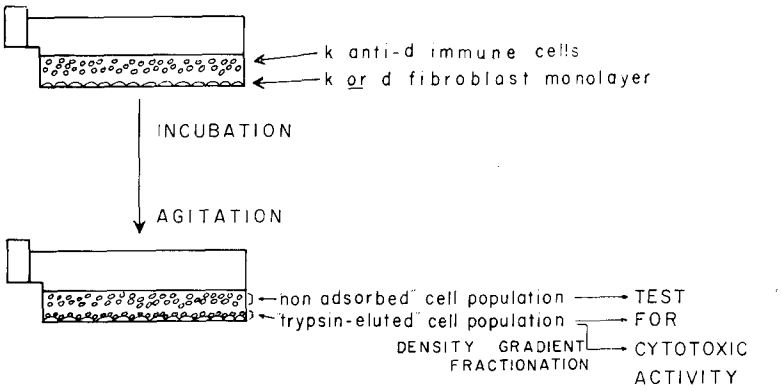

FIG. 1. Schematic representation of an experiment designed to prove specific adsorption of immune cells on fibroblast monolayers.

Gradient Fractionation of Trypsinized Cells.—Trypsin-eluted cells consist of a mixture of trypsinized fribroblasts and trypsinized spleen cells. In order to obtain purified cell populations, pools of trypsin-eluted cells from three Falcon flasks are centrifuged and resuspended in 4.5 ml of a "dense" bovine serum albumin (BSA) solution. This dense BSA solution is prepared by dissolving 28 g of purified BSA (Cohn fraction V, Koch-Light Laboratories Ltd., Colnbrook, England) in 100 ml of balanced salt solution (BSS) (balanced salt solution, Militärapoteket, Stockholm, Sweden). 4 ml of suspension of trypsin-eluted cells in dense BSA solution are put into a Beckman (Beckman Spinco, Palo Alto, Calif.) 5 ml cellulose nitrate tube and carefully overlayered with 0.5 ml of a "light" BSA solution (9 g of BSA in 100 ml of BSS). After centrifugation (g_{max} 26,000 for 30 min at 5°C), two fractions are obtained: one as a band at the interface between light and dense BSA solutions, the other as a pellet. The interface fraction is collected by puncturing the wall of the centrifuge tube 2 mm under the interface. Another puncture is made 1 cm above the bottom of the tube; the overlaying fluid is sucked off and discarded. The remaining fluid is used to resuspend the pellet. Both this pellet fraction and the interface fraction are mixed with nine times their volume of fresh culture medium, washed, resuspended in culture medium according to the desired cell concentration, and tested for cytotoxic activity.

Test for Cytotoxic Activity.—Two different types of ^{51}Cr-labeled target cells are used. When

only d target cells are needed within an experiment, use is made of P815-X2 mastocytoma cells, which exhibit a very high sensitivity to the effect of killing cells (11). 5 million mastocytoma cells in 10 ml of tissue culture medium are labeled with 200 μCi of ^{51}Cr (The Radiochemical Centre, Amersham, England) for 4 hr at 37°C. When d and other target cells are needed, use is made of secondary cultures of mouse embryonic fibroblasts, grown to confluence in Falcon flasks, labeled as for the mastocytoma cells, and trypsinized for 5 min at 37°C. Both types of target cells are then washed twice, counted, and diluted to give a final concentration of 2.5×10^4 cells/ml.

Immune spleen cell suspensions are adjusted to give a final concentration of 1.5×10^6 trypan blue excluding cells/ml, and either mixed immediately with the target cells or first incubated on fibroblast monolayers. This latter procedure leads to a decrease in cell number of about 40% (see Results). Hence, ratios of immune cells to target cells are 60:1 for immune cells tested for cytotoxicity without any preliminary incubation on monolayers, and about 35:1 for nonadsorbed immune cells after incubation on monolayers. Other ratios used in some experiments are specified in the appropriate parts of Results.

1-ml fractions of mixture of immune cells and ^{51}Cr-labeled mastocytoma cells are incubated in plastic test tubes (11 × 55 mm, Heger Plastics, Stallarholmen, Sweden) for 16–18 hr at 37°C in a 5% CO_2 atmosphere. 1-ml fractions of mixture of immune cells and ^{51}Cr-labeled fibroblasts are incubated in flat-bottomed glass tubes (10 × 50 mm, Grave, Stockholm, Sweden) for 36 hr in the same conditions. The tubes are then centrifuged. For each tube, total radioactivity and radioactivity of a sample of the supernatant are measured in a well-type scintillation counter. After substraction of the background, the results are expressed as "Release Index" (RI):

$$\text{RI} = \frac{\text{radioactivity of the supernatant}}{\text{total radioactivity}} \times 100.$$

This index expresses the amount of ^{51}Cr released from target cells into the supernatant. This release, when above the "physiological" release from target cells incubated alone, has been shown to be related to target cell death (12–14).

Each value given in the figures or in the tables is the mean of the RI of three test tubes. In the figures, vertical bars stand for the 95% confidence limits of the means, computed from the residual variance of the RI of all the tubes in a given experiment. In the tables use has been made of this variance to calculate the difference that would be significant ($P = 0.05$), in a Student's t test between two experimental groups in the experiment (15).

RESULTS

I. Incubation of a Population of Immune Cells on the Relevant Monolayer Leads to a Specific Decrease of its Cytotoxic Activity.—Immune k anti-d cells are incubated at 37°C for 1, 2, 3, or 4 hr, in Falcon flasks containing monolayers of k or d fibroblasts or no fibroblasts. At the end of the incubation period, the flasks are shaken, the nonadsorbed cells are poured off, and their cytotoxic activity tested (Fig. 2). Incubation in flasks without a monolayer leads to a progressive decrease of cytotoxic acitivity, probably due both to adsorption of cells on the plastic walls and to lack of the feeder layer effect of a fibroblast monolayer. Incubation on k fibroblasts leads to a significant early nonspecific decrease of cytotoxic activity, which has a tendency to be less pronounced with longer incubation. Incubation on d fibroblasts leads to a specific decrease of cytotoxic

activity, early, stable with time, and very marked since the release index falls almost to the level of spontaneous ^{51}Cr release by target cells in the absence of immune cells. This means that incubation of a cytotoxic immune cell population on the relevant fibroblasts leaves a nonadsorbed cell population which is virtually noncytotoxic. In this experiment, counts of viable nonadsorbed cells were made after 3 hr of incubation on the monolayers, that is when specific decrease of cytotoxicity was maximal. Results expressed as percentages of the total number of viable cells put in the flasks are: 73% for incubation in flasks

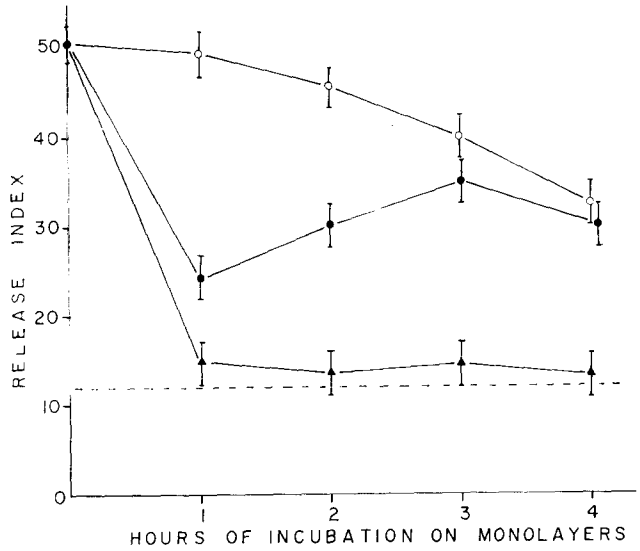

FIG. 2. Residual anti-d cytotoxic activity of nonadsorbed k anti-d immune spleen cells, after 1, 2, 3, or 4 hr of incubation at 37°C in Falcon flasks containing no fibroblast monolayer (-○-), or k (-●-), or d (-▲-) fibroblast monolayer. Cytotoxicity is expressed as Release Index. Dotted line (- -) represents Release Index of target cells alone.

without a monolayer, 48% for incubation in flasks with a k monolayer, 55% for incubation in flasks with a d monolayer.

The specific decrease of cytotoxic activity is temperature dependent. Four experiments have been done to test the effect of the temperature of incubation on monolayers on subsequent cytotoxic activity (Table I). Incubation at 4°C leads to no specific decrease of cytotoxic activity at all, or to a specific decrease of cytotoxic activity significantly less marked than that after incubation at 37°C.

After incubation, flasks are agitated mechanically before pouring off the cell suspension to be tested for cytotoxicity. Immune cells are incubated for 3 hr at 37°C in flasks containing d or k monolayers, and then subjected for 10 sec to

different arbitrary degrees of mechanical agitation. These range from one (flasks agitated very gently) to four (strong agitation leading to some foaming of the tissue culture medium). Specific decrease of cytotoxic activity remains high, regardless of the degree of agitation employed (Fig. 3).

As a standard procedure for all subsequent experiments, we have used a 3–3 hr incubation on monolayers, at 37°C, followed by "degree three" mechanical

TABLE I

The Cytotoxicity of Nonadsorbed k Anti-d Immune Spleen Cells for d Target Cells after Incubation at 37° or at 4°C on Fibroblast Monolayers

Experiment	Spontaneous release*	Cytotoxicity‡ of nonadsorbed cells after incubation on		Temperature of incubation	Specific decrease of cytotoxic activity§	Smallest significant difference‖
		k Monolayers	d Monolayers			
				°C	%	
1	20	47	24	37	85	4.6
		48	31	4	60	
2	16	33	18	37	88	4.1
		40	43	4	0	
3	17	31	22	37	72	2.3
		31	31	4	0	
4	15	55	18	37	93	3.0
		42	33	4	33	

* Spontaneous ^{51}Cr release by target cells alone.

‡ Expressed as ^{51}Cr release index.

§ If s is spontaneous release, k and d cytotoxicity of nonabsorbed cells after incubation respectively on k and d monolayers, this figure is given by the formula: $\frac{k - d}{k - s} \times 100$.

‖ Smallest difference to be significant ($P = 0.05$) in a Student's t test between two figures in this experiment.

agitation. The resulting nonadsorbed cell population is tested for cytotoxicity. Table II shows the results of 15 successive experiments of this kind. Specific decrease of cytotoxic activity is a very reproducible phenomenon; it occurs in every experiment and is always very marked.

Counts of viable nonadsorbed cells are performed after incubation on the monolayers. A considerable nonspecific adsorption is a constant feature; no more than 50–60% of the original cell input is recovered, irrespective of the *H-2* specificity of the monolayer. A super-added specific adsorption is a less constant feature; in some experiments fewer cells are recovered from d monolayers than from k monolayers; in some other experiments no significant differences in cell numbers are found, which is in sharp contrast to very significant differences in the cytotoxic activity of the same populations.

II. The Specific Decrease of Cytotoxic Activity Is Linked to Specific Adsorption of Immune Cells onto the Relevant Monolayers.—The experiments described above confirm Brondz' findings, as far as specific decrease of cytotoxic activity of nonadsorbed immune cells is concerned. However, neither Brondz' findings nor our experiments of section I prove by themselves the existence of specific surface receptors, because explanations other than specific adsorption might account for specific decrease of cytotoxic activity. Therefore, we tried to demonstrate the presence of specifically adsorbed cells, by recovering them from the monolayers.

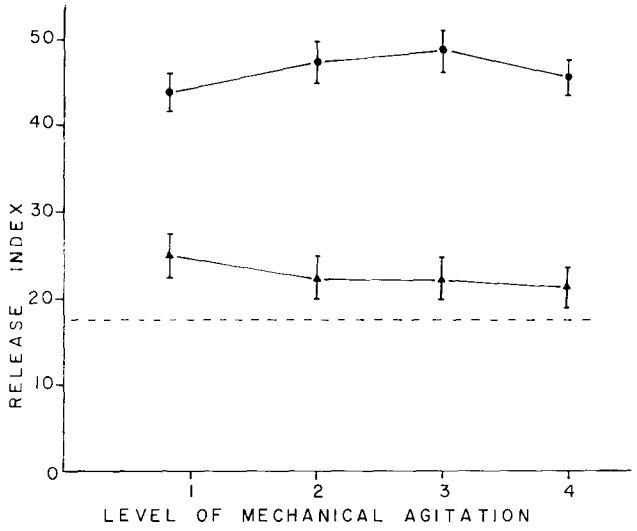

FIG. 3. Residual anti-d cytotoxic activity of nonadsorbed k anti-d immune spleen cells, after 3 hr of incubation at 37°C in Falcon flasks containing k (-●-) or d (-▲-) fibroblast monolayer. These flasks have been subjected to increasing levels of mechanical agitation, in order to try to detach putatively adsorbed killing cells. Cytotoxicity is expressed as Release Index. Dotted line (- -) represents Release Index of target cells alone.

After incubation of immune cells on monolayers, and after pouring off the nonadsorbed cell population, both the fibroblast monolayers and lymphoid cells adsorbed to them remain. Attempts to elute the lymphoid cells by mild treatments (pipetting, incubation with ethylenediaminetetraacetate [EDTA], short-time incubation with trypsin) were not successful. It is necessary to trypsinize the whole monolayers at 37°C for 30 min. The resultant cell suspension consists of lymphocytes and fibroblasts, in roughly similar concentrations, and erythrocytes. This trypsin-eluted cell population is then suspended in the same volume of medium as the nonadsorbed cell population and tested for cytotoxicity (Table III). The nonadsorbed k anti-d cell population has again lost most of its cytotoxic activity after incubation on a d monolayer, when

compared to the same cell population after incubation on a k monolayer. In contrast, the d monolayer trypsin-eluted cell population is much more cytotoxic than the k monolayer trypsin-eluted cell population. This crisscross pattern suggests that cells necessary for the killing have been specifically adsorbed on the d monolayer and are now found in the trypsin-eluted cell population.

TABLE II

The Cytotoxicity of Nonadsorbed k Anti-d Immune Spleen Cells for d Target Cells after Incubation on Fibroblast Monolayers for 3–4 Hr at 37°C

Experiment	Spontaneous release*	Cytotoxicity‡ of nonadsorbed cells after incubation on		Specific decrease of cytotoxic activity§	Smallest significant difference‖
		k Monolayers	d Monolayers		
				%	
1	15	41	30	42	4.5
2	20	39	25	74	2.6
3	18	55	23	87	3.7
4	18	46	22	86	3.1
5	18	57	20	95	4.9
6	12	31	13	97	3.6
7	14	32	18	78	2.0
8	14	25	16	82	2.1
9	13	44	16	90	2.6
10	13	23	14	90	2.7
11	17	43	18	96	3.1
12	16	26	17	90	2.5
13	17	27	16	100	2.7
14	14	58	29	66	4.6
15	15	71	32	70	3.7

* Spontaneous ^{51}Cr release by target cells alone.

‡ Expressed as ^{51}Cr release index.

§ If s is spontaneous release, k and d cytotoxicity of nonadsorbed cells after incubation respectively on k and d monolayers, this figure is given by the formula: $\frac{k - d}{k - s} \times 100$.

‖ Smallest difference to be significant ($P = 0.05$) in a Student's t test between two figures in this experiment.

However, this trypsin-eluted cell population contains both lymphoid cells and fibroblasts. Any possible role of these fibroblasts in the toxicity of the trypsin-eluted cell population (for instance, dying d fibroblasts could release some substance harmful to the labeled target cells) must be excluded. This is done by fractionation of the trypsin-eluted cell population on a discontinuous BSA gradient. The two fractions obtained are characterized as to cell type (trypsinized fibroblasts are easily distinguished on morphological grounds from trypsinized lymphocytes, which are much smaller and of smoother contour) and cytotoxic activity (Table IV). The interface fraction contains mainly

fibroblasts, the pellet fraction lymphocytes and erythrocytes. Cross-contamination is never higher than 7%. "Interface" cells are not cytotoxic, even at high ratios. "Pellet" cells are cytotoxic, and sometimes very cytotoxic, even at low ratios. This anti-d cytotoxic activity is always higher for k anti-d lymphocytes isolated from d-adsorbing monolayers than for similar lymphocytes isolated from k-adsorbing monolayers. Additional control experiments show that

TABLE III

The Cytotoxicity of k Anti-d Immune Spleen Cells for d Target Cells after Incubation on Fibroblast Monolayers. Comparison between Cytotoxicity of Nonadsorbed and of Trypsin-Eluted Cells

Experiment	Spontaneous release‖	Cells‡ tested for cytotoxicity§	Monolayers used for incubation		Smallest significant difference‖
			k	d	
1	14	nonadsorbed	32	18	2.0
		trypsin eluted	16	26	
2	13	nonadsorbed	44	16	2.6
		trypsin eluted	28	46	
3	16	nonadsorbed	26	17	2.5
		trypsin eluted	23	27	
4	14	nonadsorbed	25	16	2.1
		trypsin eluted	13	19	
5	14	nonadsorbed	18	13	2.6
		trypsin eluted	18	24	

* Spontaneous ^{51}Cr release by target cells alone.

‡ Immune spleen cells are incubated on fibroblast monolayers. Nonadsorbed cells are the cells recovered in the supernatant after agitation. Trypsin-eluted cells are the mixture of fibroblasts and adsorbed lymphocytes obtained by trypsinization of the monolayers.

§ Cytotoxicity is expressed as ^{51}Cr release index.

‖ Smallest difference to be significant ($P = 0.05$) in a Student's t test between two figures in this experiment.

this cytotoxicity is specifically directed against d target cells. Thus, there is little doubt that, among the mixture of lymphocytes and fibroblasts recovered by trypsinization, it is the lymphocytes that are specifically cytotoxic. Moreover, their cytotoxicity is higher than the cytotoxicity of an unfractionated trypsin-eluted cell population (where trypsinized fibroblasts may thus act as competitive target cells, without however completely preventing the killing of ^{51}Cr-labeled mastocytoma cells), and also is higher than the cytotoxicity of the initial immune spleen cell population (which is in line with selection, by specific adsorption, of cells involved in killing).

III. Cell Populations from "Double-Immunized" Animals Exhibit a Differential Specific Adsorption Pattern.—Immune cells from mice immunized against one type of allogeneic tumor cell bear specific receptors directed against immunizing cell surface determinants. The existence of these receptor-bearing

TABLE IV

The Cytotoxicity of k Anti-d Immune Spleen Cells for d Target Cells after Incubation on Fibroblast Monolayers. Comparison between Cytotoxicity of Nonadsorbed and of Trypsin-Eluted, Gradient Fractionated Cells

Experiment	Spontaneous release*	Cells‡ tested for cytotoxicity	Cross-contamination§	"Killing": target cell ratios	Cytotoxicity‖ of cells after incubation on		Smallest significant difference¶
					k Monolayers	d Monolayers	
			%				
1	13	nonadsorbed		45:1	38	28	2.4
		pellet	7	12:1	18	23	
		interface	4	14:1	12	12	
2	13	nonadsorbed		35:1	58	41	
		pellet	7	10:1	46	63	3.1
		interface	1	44:1	13	14	
3	14	nonadsorbed		40:1	34	19	6.1
		pellet	3	10:1	26	33	
		interface	2	36:1	15	14	
4	15	nonadsorbed		35:1	44	24	
		pellet	2	10:1	22	35	2.4
		interface	2	40:1	12	12	

* Spontaneous ^{51}Cr release by target cells alone.

‡ Immune spleen cells are incubated on fibroblast monolayers. Nonadsorbed cells are the cells recovered in the supernatant after agitation. Trypsinization of the monolayers yields a mixture of fibroblasts and adsorbed lymphocytes, which is fractionated on a discontinuous BSA density gradient. The pellet fraction consists almost exclusively of lymphocytes and erythrocytes; the interface fraction consists almost exclusively of fibroblasts.

§ Percentage of contaminating fibroblasts among pellet lymphocytes and of contaminating lymphocytes among interface fibroblasts.

‖ Cytotoxicity expressed as ^{51}Cr release index.

¶ Smallest difference to be significant ($P = 0.05$) in a Student's t test between two figures in this experiment.

cells is demonstrated by specific adsorption onto the relevant cell monolayer. A further question is what happens in animals immunized with two different types of allogeneic tumor cells, for instance in a strain mice receiving at the same time d and k tumor cells (a strain mice would be immunized against *H-2* specificity 31 by d cells, and against *H-2* specificity 32 by k cells; there is no known cross-reactivity within the *H-2* system). Two possibilities exist:

(*a*) There may be only one population of immune cells, each cell bearing both anti-d and anti-k receptors. In that case, incubation on a d monolayer would result in adsorption of these cells, leading to a decrease in both anti-d and anti-k cytotoxic activity of the nonadsorbed cells. Similarly, incubation on a k monolayer would lead to a decrease in both anti-d and anti-k cytotoxic activity.

(*b*) Alternatively, there may be two immune cell populations, one bearing

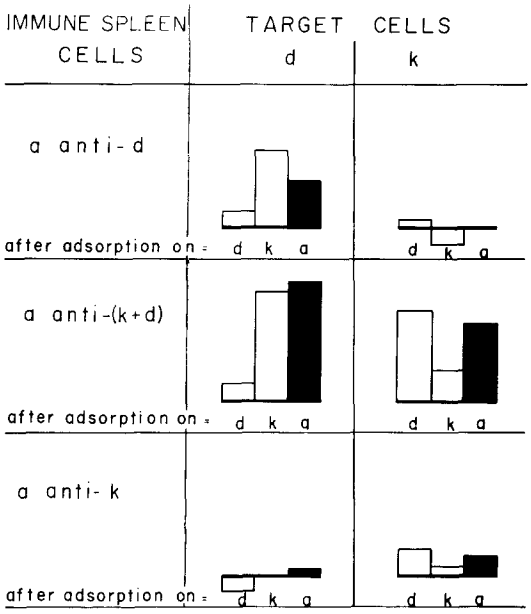

FIG. 4. Residual cytotoxic activity of spleen cells from a strain mice immunized against either d tumor cells alone, or k tumor cells alone, or a mixture of d and k tumor cells, after incubation on either a, or d, or k fibroblast monolayer. Nonadsorbed cells are tested for cytotoxicity against either d or k ^{51}Cr-labeled fibroblasts. Cytotoxicity is expressed as Release Index of experimental group minus Release Index of target cells alone. The highest column of this graph represents a cytotoxic activity of 18%.

anti-d receptors and the other bearing anti-k receptors. In that case, incubation on a d monolayer would result in adsorption of anti-d cells but not anti-k cells. There would thus be a decrease in the anti-d, but not in the anti-k, cytotoxic activity of the nonadsorbed cells. Conversely, incubation on a k monolayer would lead to a decrease in anti-k, but not in anti-d, cytotoxic activity.

Results of such an experiment are given in Fig 4. After immunization against d tumor cells, the anti-d killing activity of immune a cells can be decreased by incubation on a d monolayer, but not on an a or k monolayer. After immunization against k tumor cells, the anti-k killing activity can be decreased by incubation on a k monolayer, but not on an a or d monolayer. After simultaneous

immunization against both d and k tumor cells, incubation on a d monolayer leads to a decrease of the anti-d but not the anti-k killing activity; conversely, incubation on a k monolayer leads to a decrease of the anti-k but not the anti-d killing activity.

This experiment has been repeated with essentially similar results (Table V). We believe that it demonstrates the coexistence, in double-immunized ani-

TABLE V

The Cytotoxicity of Nonadsorbed a Anti-(d + k) Immune Spleen Cells after Incubation on d or k Fibroblast Monolayers

Experiment	Target cells*	Cytotoxicity‡ of nonadsorbed cells after incubation on		Smallest significant difference§
		k Monolayers	d Monolayers	
1‖	k	4	14	2.8
	d	17	2	
2	k	6	15	4.6
	d	24	8	
3¶	k	14	31	2.6
	d	22	5	
4	k	9	21	3.1
	d	26	4	

* Target cells are d and k ^{51}Cr-labeled fibroblasts.

‡ Expressed as release index minus spontaneous release of target cells alone, thus taking into account slight differences in spontaneous release that may occur between d and k fibroblasts used in a given experiment. Spontaneous ^{51}Cr release of fibroblasts after 36 hr is usually about 40%.

§ Smallest difference to be significant ($P = 0.05$) in a Student's t test between two figures in this experiment.

‖ Complete results of this experiment are shown in Fig. 4.

¶ Whereas each of the other experiments is done with a pool of spleen cells from two double-immunized mice, experiment three is done with spleen cells taken from only one double-immunized mouse.

mals, of two populations of immune cells bearing either one or the other type of specific receptor.

DISCUSSION

We have incubated immune spleen cells on fibroblast monolayers bearing the immunizing histocompatibility antigens, or on control fibroblast monolayers not bearing these antigens. Incubation on the former monolayers leads to a population of nonadsorbed cells having far lower specific cytotoxic activity than the initial immune cell population. Elution experiments prove that this

specific decrease of cytotoxic activity is because of specific adsorption, onto the monolayers, of cells necessary for the killing. Specific adsorption of cells indicates specific receptors at the surface of these cells.

Several questions may be asked about these receptor-bearing cells. A first question is their frequency. It has been shown that, in at least some experiments, a very significant specific decrease of cytotoxic activity is not accompanied by any detectable specific decrease in the number of nonadsorbed cells. As specific decrease of cytotoxic activity is linked to adsorption of receptor-bearing cells, it follows that the proportion of these cells among immune spleen cells must be small. Any precise estimate of the proportion of receptor-bearing cells by adsorption techniques has to overcome two difficulties, not to speak of inacurracy of cell counts. First, many nonrelevant cells are adsorbed. This nonspecific adsorption reaches 40–50% of the total initial number of cells in our experiments and can be estimated by using the appropriate controls. Second, some receptor-bearing cells may not be adsorbed. The proportion of killing cells among immune rat lymph node cells has been estimated by other techniques to be around 1–2% (6). However, as will be discussed further, it is not possible at present to say that killing cells and receptor-bearing cells are the same.

Another question is whether, in animals immunized against cells from two tumors with different histocompatibility antigens, there is only one population of immune cells each bearing both types of specific receptor, or two populations of cells each of which would bear only one type of receptor. The experiments described in this paper demonstrate the existence of two populations of cells in double-immunized animals, showing that a majority if not all the receptor-bearing cells bear only one type of receptor. This conclusion has also been suggested by some less direct experiments published very recently by Brondz and Snegiröva (16). B anti-a lymphocytes were adsorbed on mixtures of d and k macrophages used in various proportions; the results indicated that these b anti-a lymphocytes represent a mixture of two populations, presumably bearing receptors directed against either the d or k components of a. It should be stressed that one cannot exclude the possibility of the existence of a minority of cells, each bearing both types of receptor. In other words, receptor homogeneity per cell may be the rule, but receptor heterogeneity for some cells cannot be excluded.

The coexistence, in double-immunized animals, of cells bearing either one or the other type of specific receptor is relevant to a consideration of the mechanism by which cells acquire these receptors. Several mechanisms could be considered. The receptors may be made by one cell type, excreted, and adsorbed onto other cells, just as cytophilic antibodies are adsorbed onto macrophages (17). In double-immunized animals two different types of receptor would be made, excreted, and adsorbed; random adsorption on cells of the two different types of receptor would lead to receptor heterogeneity for most of the receptor-

bearing cells. Our results show that there is, on the contrary, at least a majority of cells with receptor homogeneity. Therefore, one can exclude random adsorption as a mechanism of acquisition of receptors by cells. Nonrandom adsorption would imply specific differences between the two populations of cells before adsorption of the two different types of specific receptors; this mechanism is *ipso facto* redundant as to specificity and therefore highly improbable. One might still argue that adsorption of receptors is taking place in the form of antigen-antibody complexes, which would be created in the mixture of immune cells and cells carrying the immunizing antigens. Experiments in vitro where normal lymphocytes are incubated with antibody-coated target cells give results suggesting that antigen-antibody complexes might be adsorbed onto lymphocytes, thereby converting these cells into "specific" killer cells (Perlmann, P., personal communication). The presence of nonthymus-derived lymphocytes (B cells) would be required for this interpretation of our results to be satisfactory, as these are the cells which actively secrete antibodies (18). Measures to exclude B cells from the present system have, however, completely failed to demonstrate any "positive" effect of B cells on killing.[1] In conclusion, since adsorption of receptors onto cells seems unlikely, we consider that these receptors are made by the cells that bear them.

The role of the receptor-bearing cells seems clear as far as specificity of killing is concerned. Cell killing requires close contact between target cells and at least some of the cells involved in the killing process, since interposition of a Millipore filter (Millipore Corp., Bedford, Mass.) between killing cells and target cells prevents killing (5, 6). Microcinematographic studies have given direct evidence of contacts between target cells and killing cells, especially through the lymphoid cell uropod region (19). These facts, the requirement for specificity, and the presence of specific receptors at the surface of cells involved in killing make it likely that an important step of the killing process, at least in this type of experimental system, is the formation of a complex between a specific receptor of a receptor-bearing cell and a surface antigen of a target cell. A further argument in favor of this view is the fact that antibodies directed against target cell histocompatibility antigens may inhibit the cytotoxic effect of killing cells (20, 6, 21, 22) perhaps by competing with the specific cell surface receptors for the target cell antigens. Final proof of the necessity of this step for the cytotoxic process would be given if specific blocking of the surface receptors of lymphoid cells, by for instance solubilized histocompatibility antigens, led to inhibition of cytotoxicity.

Little information is available about the receptor–surface antigen complex. It is destroyed by trypsinization; the trypsin-eluted cell population, which initially consists of lymphoid cells adsorbed on fibroblasts, can be fractionated after trypsinization into almost pure populations of lymphoid cells on one hand and of fibroblasts on the other hand. It may be that trypsinization affects both elements of the complex. Specific receptors may be altered, since tryp-

sinization of immune cells temporarily inhibits their cytotoxity (11); our results with trypsin-eluted cells confirm the reversibility of this inhibition during the 18 hr of the cytotoxic test. Target cell surface antigens may also be altered. However, trypsinization may alter many cell surface structures, interfering in a completely "unspecific" way with the build up or the maintenance of a receptor–surface antigen complex.

Another experimental observation is that specific adsorption of receptor-bearing cells is greatly reduced at 4°C. However, adsorption of cells to cells may involve many events (some certainly thermodependent, such as cell positioning or cell membrane rearrangements) other than the actual formation of a complex between two cell surface structures; hence, this observation may not be relevant to the temperature requirements of the formation of a receptor–surface antigen complex.

Receptor-bearing cells are necessary for immune killing because their removal by specific adsorption prevents it. However, we do not know if these receptor-bearing cells are necessary only to recognize the target cells, or to recognize and also kill them. What follows recognition in the chain of events leading to target cell death still remains conjectural. An interesting observation is that nonrelevant target cells may be killed in the vicinity of immune lymphocytes engaged in the specific killing of relevant target cells (23–26). Another fact is that lymphoid cells, preimmunized against a given antigen, may release some nonspecific cytotoxic factor(s) when confronted in vitro with this antigen (27–29). Thus, in cell-mediated specific cytotoxicity under certain experimental conditions, actual killing could be because of nonspecific factor(s). These factors, or other substances which cause target cell death, may be released from, or carried by, the receptor-bearing cells themselves. The receptor-bearing cells would thus both recognize and kill the target cells. Alternatively, other cells also may be involved. The receptor-bearing cells would then only recognize (and possibly also sensitize) the target cells, actual killing being performed by other cells or their products.

Fractionation procedures to obtain "pure" populations of thymus-derived lymphocytes (T cells), nonthymus-derived lymphocytes (B-cells), or other cells would be necessary for a further understanding of the processes involved in specific cell-mediated cytotoxicity. Preliminary experiments, using such fractionation procedures combined with the specific adsorption-elution techniques described in this paper, give further information about which cells are involved in specific killing, and, moreover, suggest the feasibility of analysis of T cell specific receptors.[1]

SUMMARY

Spleen cells from mice immunized with allogeneic tumor cells are incubated on different fibroblast monolayers. The nonadsorbed cells are tested for cytotoxicity against ^{51}Cr-labeled target cells. The cytotoxicity of nonadsorbed cells is

much lower after incubation on fibroblasts syngeneic to the immunizing tumor cells than after incubation on fibroblasts syngeneic to the immune cells. This specific decrease of cytotoxic activity depends on the duration and temperature of incubation on monolayers. After incubation the monolayers are trypsinized and pure populations of adsorbed lymphocytes isolated by density gradient fractionation. The cytotoxicity of such trypsin-eluted, gradient-purified lymphocytes is much higher when these lymphocytes are isolated from fibroblasts syngeneic to the immunizing tumor cells than when they are isolated from fibroblasts syngeneic to the immune cells. These experiments demonstrate specific adsorption of immune cells onto fibroblasts carrying the immunizing antigens, and thus prove the existence of specific receptors at the surface of these immune cells.

Spleen cells from mice immunized with two types of allogeneic tumor cells bearing different *H-2* antigen alleles are incubated on different fibroblast monolayers. The results of such experiments show a differential specific adsorption pattern, suggesting independent adsorption of two populations of immune cells bearing receptors directed against either one or the other immunizing *H-2* antigen. The existence of at least a majority of cells, each of which is homogeneous as to the specificity of its receptors, makes it likely that specific receptors are synthetized by the cells that bear them. The role of specific receptor-bearing cells in the killing process is discussed.

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