CELLS MEDIATING SPECIFIC IN VITRO CYTOTOXICITY

II. Probable Autonomy of Thymus-Processed Lymphocytes (T Cells) for the Killing of Allogeneic Target Cells*

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Lymphocytes can be divided into two populations, a population of cells that have been processed by the thymus (T cells)[1] and a population of cells that have not been processed by the thymus (B cells) (1). T cells collaborate with B cells for the induction of antibody synthesis in response to some antigens (2, 3). The specificity of their participation (4, 5) strongly suggests the existence of specific receptors for antigen on the T cell surface. However, direct evidence for this is still scarce. Fractionation techniques allowing the detection of such receptors on B cells have so far yielded completely negative results with T cells (6). However, positive results have been reported in experiments where delayed hypersensitivity reactions were "blocked" with anti-light chain antisera (7, 8) and in experiments where in vivo T cell "helper" activity was specifically suppressed by an ^{125}I-labeled antigen "suicide" technique (9, 10).

T cells are also involved in another type of immunological reaction. Lymphoid cells from animals immunized against allogeneic cells are able to kill these cells in vitro (11, and reference 12 for review). "Cell killing," throughout this paper and unless otherwise stated, refers to this type of in vitro killing of target cells by allogeneic immune lymphoid cell suspensions. Cell killing, in the mouse system, may take place when lymphoid cell populations consist mostly of T cells (13, 14) and can be abolished by anti-θ antiserum in the presence of complement (15). This indicates that T cells are involved in cell killing, but does not formally exclude some participation of other cells. Cells involved in killing have also been characterized in another way. Incubation of a killing cell population on monolayers of cells of the same genotype as the cells used for immunization results in a cell population of greatly reduced killing efficiency

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[1] *Abbreviations used in this paper:* B cells, nonthymus-processed lymphocytes; BSA, bovine serum albumin; EAC, erythrocytes sensitized with antibody and complement; MEM, Eagle's minimal essential medium; PVP, polyvinylpyrrolidone; R.I., release index; T cells, thymus-processed lymphocytes.

(16, 17). Elution experiments have shown that this is due to specific adsorption on the monolayers of cells involved in killing (17). This established the existence of cells, involved in killing, bearing specific receptors at their surface.

The demonstration that these receptor-bearing cells are T cells would be useful, both for a better understanding of the mechanism of killing and for further studies on T cell receptors. This demonstration could be achieved by showing, for instance, that *only* T cells are involved in killing in this system. Thus we would prove that receptor-bearing cells, which are involved in killing in this system, are T cells.

Materials and Methods

General Design of Experiments.—First, involvement of T cells in killing was confirmed in experiments where the killing capacity of immune spleen cells was suppressed by incubation with anti-T cell antiserum plus complement. Then, we investigated the possibility that only T cells are involved, by testing the cytotoxicity of cell populations as deprived as possible of B cells. Use was made of (*a*) immune spleen cells deprived of B cells by passage through a column of plastic beads coated with an anti-mouse immunoglobulin antiserum, (*b*) "educated" thymus cells, i.e. normal thymus cells sensitized by injection into a semiallogeneic recipient (13, 14), and finally (*c*) cells resulting from a combination of both techniques, i.e., educated thymus cells further purified by passage through a column. Each of these cell populations was tested for cytotoxicity against relevant target cells (that is, on target cells bearing determinants against which the lymphocytes were immunized), for cytotoxicity against non-relevant target cells (to check the specificity of killing), and also for specific adsorption on fibroblast monolayers of cells involved in killing (to check for the presence of receptor-bearing cells).

Some of the methods (tissue culture conditions, preparation of immune spleen cell suspensions, the test for specific adsorption on fibroblast monolayers, the labeling of target cells, and the test for cytotoxic activity) were described in detail in the first paper of this series (17) and will thus only be described briefly.

Mice.—1–2-month old mice from the following inbred strains, and their hybrids, were utilized: A/Sn ($H\text{-}2^a$, further quoted as *a*), BALB/c and DBA/2 ($H\text{-}2^d$, further quoted as *d*), CBA and C3H ($H\text{-}2^k$, further quoted as *k*). We have not detected any effect linked to non-*H-2* antigens in this system.

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

Preparation of Killing Cells.—

Immune spleen cells: *K* mice received a first intraperitoneal injection of 10^6 P815-X2 cells (methyl cholanthrene-induced *d* mastocytoma cells) and 2 wk later a second intraperitoneal injection of 3×10^7 P815-X2 cells. 9–15 days after the second injection, the mice were killed and spleen cell suspensions were prepared and adjusted to the desired concentration.

Educated thymus cells: Normal thymus cell suspensions were prepared in Eagle's minimal essential medium (MEM) by passage through a stainless steel net (60 mesh). The cells were centrifuged once and resuspended in MEM to obtain a concentration of 2×10^8 cells/ml. F_1 hybrids (for instance, $a \times d$ mice if the donors of thymus cells were *a*) were given a 700 R irradiation as described previously (18) and received intravenously 0.5 ml of the thymus cell suspension. These recipient mice were sacrificed 5 days later and a suspension of their spleen

cells was prepared and adjusted to the desired concentration. Such a suspension contains cells which are 92–95% of donor origin (18).

Preparation and Use of a Rabbit Anti-Mouse T Cell Antiserum.—Three rabbits each received 10^9 BALB/c thymus cells intravenously, in two injections 3 wk apart. The animals were bled individually 1 wk after the last immunization. Each serum was then adsorbed with BALB/c myeloma cells (5A = Ig_{2a}, 603 = IgA) using a serum to packed cell ratio of 3:1, at 4°C for 1 hr. This procedure was repeated six times and followed by one adsorption using pools of lymph node, bone marrow, and spleen cells derived from thymectomized, lethally X-irradiated, bone marrow–protected animals. One of these three sera was thus rendered specific for T cells according to the following criteria: (*a*) It was cytotoxic for T cells in cytotoxicity tests in vitro at a dilution of 1:10,000 (killing 100% of thymocytes, 80% of lymph node cells from 1:20 up to 1:10,000). It would not kill B cells if diluted above 1:10 (e.g. cytotoxicity was lost at a dilution of 1:5 against hemolytic plaque-forming cells and at a dilution of 1:9 against spleen cells from thymectomized, lethally X-irradiated, bone marrow–protected mice). (*b*) Normal spleen cells were incubated for 1 hr at 37°C with the antiserum at a dilution of 1:100, in the presence of rabbit complement at a dilution of 1:40. The cells were subsequently washed and transferred into 800 R X-irradiated syngeneic mice together with 4×10^8 horse red blood cells, a "thymus-dependent antigen" (19), and 1 μg of polyvinylpyrrolidone (PVP, average mol wt 360,000, Fluka A.G., Basel, Switzerland), a "thymus-independent antigen" (20). The animals were bled after 10 days and each serum was analyzed for hemolytic antibodies against horse erythrocytes and also for antibodies against PVP using a modified precipitation assay (20). In the animals which had received antiserum-treated cells, the level of antibodies directed against horse erythrocytes was reduced by more than 90%, whereas the level of antibodies directed against PVP was increased by a factor of 1.5. This is in agreement with the findings of other workers on the effect of anti-lymphocyte antisera on the humoral antibody synthesis against thymus-independent antigens (21, 22).

In the present experiments, immune spleen cells were incubated for 1 hr at 37°C with this anti-T antiserum at a dilution of 1:100. Normal rabbit serum, at a dilution of 1:40, was added to some of the experimental groups as a source of complement. The immune spleen cells were washed at the end of the incubation period and then tested for viability with trypan blue and for cytotoxic activity.

Filtration of Cell Suspensions through Columns Coated with Anti-Mouse Immunoglobulin (Ig) Antiserum.—Degalan V26 beads (Degussa Wolfgang A.G., Hanau am Main, Germany) were rinsed extensively in sterile distilled water. Mouse gamma globulin (0.5% in phosphate-buffered saline, 0.15 M, pH 7.4) was added to the beads which were then kept at 45°C for 1 hour followed by overnight incubation at 4°C. Mouse gamma globulin was obtained by precipitation of normal mouse serum with 40% ammonium sulfate, followed by filtration through G-25 Sephadex columns (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) to remove the ammonium sulfate. No determination of the degree of contamination by other serum proteins was made. After overnight incubation, the beads were poured into a glass column (1.5 × 30 cm, Pharmacia) and washed with 200 ml of sterile saline. The column was then filled with a polyvalent rabbit anti-mouse Ig antiserum at a dilution of 1:5 in saline and left for another 2 hr at 4°C. The antiserum contains antibodies reactive against all known heavy and light mouse Ig chains (23). It was used in such an excess that less than 5% of its anti-Ig activity was adsorbed onto the Ig-coated beads. The column was washed with 100 ml of sterile tissue culture medium and was then ready for use. It would now contain anti-Ig molecules, many of which would bind to the bead-attached Ig through only one of their antigen-binding sites, leaving the other site free to react with cell membrane–bound Ig. The fractionation of the cells was carried out using a flow rate of 2–3 ml/min under conditions previously described (24). Cell recovery was about 20%. Control columns were coated with normal rabbit serum only.

The following tests were used to study the population changes after filtration through the anti-mouse Ig antiserum-coated columns: (*a*) A cytotoxic assay with the specific rabbit anti-T antiserum described above, using the trypan blue exclusion method (25), was carried out using serum concentrations giving plateau levels of cytotoxicity. (*b*) A "rosette" cell test detecting complement-reactive lymphocytes, using anti-sheep erythrocyte rabbit IgM plus mouse complement in sublytic concentrations to make sheep erythrocytes into EAC (erythrocytes sensitized with antibody and complement) (26), was used to detect B cells according to Bianco et al. (26). (*c*) Another rosette cell test, using sheep erythrocytes coated with anti-sheep erythrocyte rabbit IgG in the absence of complement, was used to detect resting B cells and also activated T cells according to Yoshida and Andersson.[2] (*d*) An indirect fluorescent antibody technique, using at first incubation with the polyvalent rabbit anti-mouse Ig antiserum followed by washing of the cells and addition of a sheep anti-rabbit Fab fluorescein-labeled antiserum (Statens Bakteriologiska Laboratorium, Stockholm, Sweden), was carried out as previously described (27) using antiserum concentrations giving plateau levels of percentages of positive cells. This test detects only B cells according to Raff (28).

Test for Specific Adsorption.—Suspensions of killing cells in 3 ml of tissue culture medium were incubated for 4 hr at 37°C in small plastic flasks (25 cm^2, tissue culture flasks, Falcon Plastics, Div. of B-D Laboratories, Inc. Los Angeles, Calif.) containing confluent fibroblast monolayers. *K* anti-*d* immune spleen cells were incubated at a concentration of 3×10^6 cells/ml on either *k* or *d* fibroblast monolayers. *K* anti-*d* or *a* anti-*d* educated thymus cells were incubated, at a concentration of 4.5×10^5 cells/ml, also on either *k* or *d* fibroblast monolayers. (*K* anti-*d* and *a* anti-*d* cells recognize determinants on *d* fibroblasts that *k* fibroblasts do not possess.) After incubation, flasks were agitated and nonadsorbed cells were tested for cytotoxicity. Nonadsorbed cells from the *d* monolayer-containing flasks are far less cytotoxic for *d* cells than nonadsorbed cells from the *k* monolayer-containing flasks, which is due to specific adsorption of anti-*d* cells, involved in killing, onto *d* monolayers (17).

Test for Cytotoxic Activity.—Two different types of ^{51}Cr-labeled target cells were used, either P815-X2 mastocytoma cells (most frequently), or mouse embryo fibroblasts for experiments involving both *d* and *k* target cells. The cytotoxic test was carried out in test tubes, each of which contained, in a volume of 1 ml of tissue culture medium, 2.5×10^4 ^{51}Cr-labeled target cells and varying amounts of immune spleen cells or educated thymus cells. The corresponding ratios of killing cells to target cells are specified in the appropriate parts of the Results section. The tubes were incubated for 16–18 hr at 37°C in a 5% CO_2 atmosphere (24–48 hr when fibroblasts were used as target cells). They were then centrifuged, and samples of the supernatants were transferred to other tubes. Radioactivity of the initial tubes and of the tubes containing supernatant samples was measured in a well-type scintillation counter. After subtraction of the background, the results were computed and finally presented as the release index (R.I.), which expresses the amount of ^{51}Cr released from the target cells into the supernatant, for each test tube:

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

Each value given in the figures or in the tables is the mean of the R.I. of three test tubes. In the figures, vertical bars stand for the 95% confidence limit of the means, computed from the residual variance of the R.I. of all the test 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.

Requirement for Sterility.—There was practically never any microbial infection in experiments using immune spleen cells as killing cells. The situation was different with educated

[2] Yoshida, T., and B. Andersson. Manuscript in press.

thymus cells and with column-purified cells. Thymus cells are educated in irradiated recipients, where infections are not uncommon and can be carried over with educated cells to the test tubes. Microbial growth can occur during the ^{51}Cr release test in spite of the presence of antibiotics in the culture medium. We observed, in some experiments, a very high level of cytotoxicity even at very low ratios and/or on irrelevant target cells. This could be traced to infection in the cytotoxicity test tubes, inapparent at the time of reading of the cytotoxicity test but obvious after a few more hours at 37°C. We would like to stress that an infection may very much alter the results of the cytotoxicity test, even if this infection is not yet macroscopically evident at the time of reading. Specificity controls and/or sterility controls are thus strictly necessary in these experiments where there is any significant risk that suspensions of killing cells may contain microorganisms. All the experiments with educated thymus cells described in this paper had controls either for specificity of killing or for specificity of adsorption. Column-purified cells may lead to infection because of difficulties in sterilizing the Degalan bead columns. All the experiments with column-purified cells described in this paper had sterility controls (cell suspensions maintained at 37°C for at least 48 hr) and some experiments also had specificity controls.

RESULTS

Preliminary experiments were set up in order to confirm that T cells are involved in this type of in vitro killing. *K* anti-*d* immune spleen cells were incubated, in the presence or in the absence of added complement, with a rabbit antiserum specific for mouse T cells at the dilution used. These cells were subsequently tested for cytotoxicity against *d* target cells. Incubation with the anti–T antiserum in the absence of complement resulted in a slight increase in cytotoxicity (Table I), which is consistent with the finding that antibodies directed against the killing cells may increase their cytotoxicity (29, 30), and will be discussed later in connection with other results. Incubation with the anti–T antiserum in the presence of complement resulted in a decrease of trypan blue viability of the immune spleen cells of about 50% and in a complete or almost complete suppression of their cytotoxicity (Table I). This confirms analogous results obtained with anti-θ antiserum and complement (15) and shows that T cells are involved in killing. The question whether only T cells are involved is investigated below.

Another set of preliminary experiments explored the capacity of columns of beads coated with anti–mouse Ig antiserum to selectively retain B cells. Various populations of lymphoid cells were allowed to filter through such columns. The control and the passed cells were then analyzed with regard to population characteristics. The results are shown in Table II and clearly indicate that the anti–mouse Ig antiserum–coated columns have a very marked and selective capacity to retain cells with membrane characteristics of B lymphocytes. When using spleen cells, an almost complete retention of B lymphocytes was obtained yielding a virtually pure T cell population. Filtration of educated thymus cells through the columns would seem to be an even more stringent way of producing cell populations devoid of B lymphocytes. It should be added that any antigen-antibody complex on the bead columns would not retain B lymphocytes, e.g., a column coated with bovine serum albumin (BSA) and rabbit anti-BSA anti-

serum would not show any selective retention of B lymphocytes.[3] Furthermore, using γ1 mouse myeloma proteins plus specific rabbit anti-mouse γ1 antiserum for coating columns in the above described manner, a selective retention of γ1 precursor cells was recorded as measured in an in vivo transfer system.[3] It would thus seem clear that the receptors for antigen-antibody complexes demonstrable on B mouse lymphocytes would not be responsible for function of these columns.[4]

Experiments with Immune Spleen Cells Purified by Filtration through a Column Coated with Anti-Mouse Ig Antiserum.—Immune spleen cells were tested for cytotoxicity, either directly or after filtration through a column coated with

TABLE I

The Effect of Rabbit Anti-T Antiserum, with or without Complement, on the Cytotoxic Activity of Immune Spleen Cells

Experiment No.	Spontaneous release*	Cytotoxicity‡ of immune spleen cells after incubation for 1 hr at 37°C with				Smallest significant difference¶
		—	R anti-T§	C'‖	R anti-T + C'	
1	22	38	N.D.	40	19	2.2
2	16	21	28	28	15	3.4
3	13	40	50	44	16	2.7
4	16	42	49	42	24	2.5

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

‡ Expressed as ^{51}Cr release index.

§ Heat inactivated rabbit anti-T antiserum at a dilution of 1:100. N.D. = not done.

‖ Normal rabbit serum, as a source of complement at a dilution of 1:40.

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

normal rabbit serum or with a rabbit antiserum directed against mouse immunoglobulins. Fig. 1 shows that the cytotoxicity of unpurified cells was lower than the cytotoxicity of cells purified by filtration through a column coated with normal serum, which was in turn lower than the cytotoxicity of cells purified by filtration through a column coated with the antiserum. Table III shows the results of experiments comparing the cytotoxicity, at a ratio of 20 immune spleen cells to 1 target cell, of unpurified cells and of cells purified by filtration through an antiserum-coated column. It is clear that the removal of B cells obtained by column filtration does not lead to any decrease of cytotoxic activity. On the contrary, the resulting T cell–enriched cell population is consistently more cytotoxic than the original immune spleen cell population.

This increase in cytotoxic activity could be nonspecific, in the sense that column-purified cells might have acquired the ability to kill any target cell ir-

[3] Wigzell, H., C. S. Walters, and V. S. Schirrmacher. Manuscript submitted for publication.

[4] Basten, A., et al. Manuscript submitted for publication.

respective of its histocompatibility antigens. That this is not the case is shown in Table IV. *K* anti-*d* immune spleen cells killed *d* target cells much more efficiently than *k* target cells. Purification of those immune spleen cells by filtration through an antiserum-coated column led to a cell population endowed with a higher, but still specific, cytotoxic activity.

TABLE II

The Capacity of Columns of Beads Coated with Anti-Ig Antiserum to Selectively Retain Subpopulations of Lymphoid Cells

Experiment No.	Cells*	EA-reactive cells‡	EAC-reactive cells§	T cells‖	Ig cells¶
		%	%	%	%
1	S,C	32.1	56.5	38	N.D.
	S,PI	0.3	2.6	98	N.D.
2	S,C	N.D.	N.D.	45	N.D.
	S,PI	N.D.	N.D.	95	N.D.
	S,PNS	N.D.	N.D.	51	N.D.
3	S,C	66	57	34	N.D.
	S,PI	22	2.2	94	N.D.
	S,PNS	56	49	41	N.D.
4	S,C	N.D.	N.D.	41	48
	S,PI	N.D.	N.D.	96	0.8
5	ET,C	22	0	96	N.D.
	ET,PI	5.6	0	97	N.D.

* S = spleen cells; ET = educated thymus cells; C = control cells; PI = passed through a column coated with rabbit anti-mouse Ig antiserum; PNS = passed through a column coated with normal rabbit serum.

‡ Cells forming "rosettes" with sheep erythrocytes coated with anti-sheep erythrocyte rabbit IgG.

§ Cells forming rosettes with sheep erythrocytes coated with anti-sheep erythrocyte rabbit IgM plus mouse complement in sublytic concentrations.

‖ Cells judged as T lymphocytes as assessed by specific killing with a rabbit anti-T antiserum plus complement.

¶ Cells showing demonstrable membrane fluorescence using a rabbit anti-mouse Ig antiserum followed by a sheep anti-rabbit Ig antiserum.

Cytotoxic immune spleen cells can be specifically adsorbed on the relevant fibroblast monolayer (17). Fig. 2 shows that the same phenomenon was also observed with column-purified immune spleen cells.

Experiments with Educated Thymus Cells, Unpurified or Purified by Filtration through a Column Coated with Anti-Mouse Ig Antiserum.—It has previously been demonstrated that educated thymus cells are cytotoxic in vitro (13, 14). Additional experiments showed (Table V) that the level of cytotoxicity can be very different from one experiment to another and also that cytotoxicity can be

demonstrated even at a ratio of 1 killing cell to 1 target cell. Educated thymus cells were purified by filtration through a column coated with anti-mouse Ig

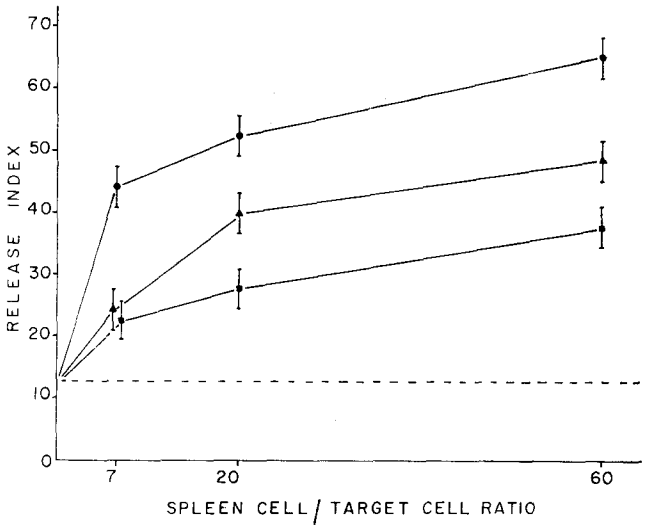

FIG. 1. Cytotoxic activity of *k* anti-*d* immune spleen cells either unpurified (—■—) or purified by filtration through a column of beads coated either with normal rabbit serum (—▲—) or with rabbit anti-mouse Ig antiserum (—●—). Cytotoxicity is expressed as Release Index. Dotted line (-----) represents R.I. of target cells alone.

TABLE III

The Cytotoxicity of Immune Spleen Cells Purified or not by Filtration through a Column of Beads Coated with Anti-Mouse Ig Antiserum

Experiment No.	Spontaneous release*	Cytotoxicity‡ of immune spleen cells§		Smallest significant difference‖
		Unpurified	Purified	
1	13	27	53	4.7
2	11	20	43	3.6
3	14	28	56	6.1
4	15	30	45	2.4
5	14	25	43	4.0

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

‡ Expressed as ^{51}Cr release index.

§ Cell concentrations were adjusted to give ratios of 20 unpurified or purified trypan blue excluding immune spleen cells to 1 target cell.

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

antiserum. Their cytotoxic activity was not depressed by this purification; except for one experiment, column-purified cells were as cytotoxic or more cytotoxic than unpurified cells (Table VI).

The cytotoxicity of educated thymus cells was specific (Table VII). *K* thymus cells educated against *d* cells were more cytotoxic to *d* target cells than *d* thymus cells educated against *k* cells. *K* or *a* strain thymus cells educated against *d* cells

TABLE IV

The Specificity of the Cytotoxicity of Immune Spleen Cells, either Unpurified or Purified by Filtration through a Column of Beads Coated with Anti-Mouse Ig Antiserum

Killing cells*	Target cells‡	Cytotoxicity§ at the following ratios of killing cells to target cells		
		60:1	20:1	7:1
Unpurified	*d*	26	17	8
	k	9	2	1
Purified	*d*	37	21	10
	k	6	2	1

* Killing cells were *k* anti-*d* immune spleen cells.

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

§ Expressed as Release Index minus Spontaneous Release by target cells alone. Spontaneous ^{51}Cr release after 36 hr was 40% for *d* fibroblasts and for *k* fibroblasts. The smallest difference to be significant ($P = 0.05$) in a Student's *t* test between two figures in this experiment was 2.8.

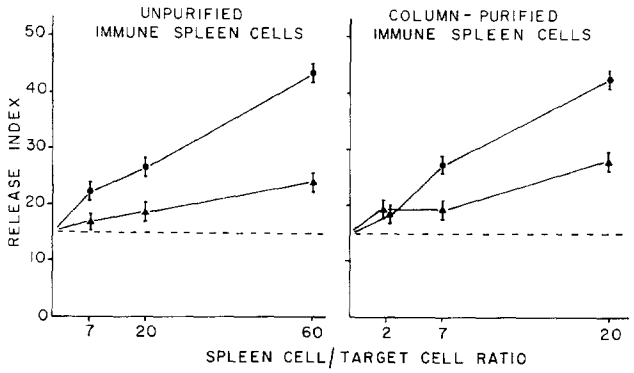

FIG. 2. Residual anti-*d* cytotoxic activity of nonadsorbed *k* anti-*d* immune spleen cells after 4 hr of incubation at 37°C in Falcon flasks containing *k* (—●—) or *d* (—▲—) fibroblast monolayers. Before this incubation, immune spleen cells had been purified or not by filtration through a column of beads coated with anti-mouse Ig antiserum. Cytotoxicity is expressed as Release Index. Dotted line (-----) represents R.I. of target cells alone.

were more cytotoxic to *d* target cells than to *k* target cells. Column-purified educated thymus cells also killed in a specific way. In one experiment, cytotoxicity of *k* anti-*d* column-purified educated thymus cells was 2 under spon-

taneous release when tested on *k* fibroblasts, and 8 above spontaneous release when tested on *d* fibroblasts, at a ratio of 10 killing cells to 1 target cell.

Educated thymus cells involved in killing could be specifically adsorbed on the relevant monolayer. Fig. 3 shows the result of an experiment comparing

TABLE V

The Cytotoxicity of Educated Thymus Cells

Experiment No.	Spontaneous release*	Cytotoxicity‡ at the following ratios of killing cells§ to target cells				Smallest significant difference‖
		9:1	3:1	1:1	0.3:1	
1	13	52	44	27	17	2.9
2	10	33	21	14	12	1.6
3	8	29	15	12	12	2.1

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

‡ Expressed as ^{51}Cr release index.

§ Killing cells were *a* anti-*d* educated thymus cells.

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

TABLE VI

The Cytotoxicity of Educated Thymus Cells Purified or Not by Filtration through a Column of Beads Coated with Anti-Mouse Ig Antiserum

Experiment No.	Spontaneous release*	Cytotoxicity‡ of educated thymus cells§		Smallest significant difference‖
		Unpurified	Purified	
1	15	55	41	2.7
2	14	40	72	6.6
3	12	29	67	4.5
4	16	62	61	6.4

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

‡ Expressed as ^{51}Cr release index.

§ Educated thymus cells were *a* anti-*d* for experiments 1–3 and *k* anti-*d* for experiment 4. Cell concentrations were adjusted to give ratios of 15 unpurified or purified trypan blue excluding educated thymus cells to 1 target cell.

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

specific adsorption of immune spleen cells and of educated thymus cells. Educated thymus cells were usually less "specifically adsorbable" than immune spleen cells, under the conditions of these experiments. Some of the parameters governing the extent of specific adsorption of educated thymus cells are presently under study. Also column-purified educated thymus cells involved in killing could be specifically adsorbed on the relevant monolayer. In one experiment where spontaneous release was 12, cytotoxicity of *k* anti-*d* educated thymus cells after incubation respectively on *k* or *d* fibroblast monolayers was

29 and 24 for unpurified, and 67 and 50 for column-purified cells, thus demonstrating similar levels of specific adsorption.

TABLE VII

The Specificity of the Cytotoxicity of Educated Thymus Cells

Experiment No.	Educated thymus cells	Target cells*	Cytotoxicity ‡	Smallest significant difference§
1	*k* anti-*d*	*d*	17	4.5
	d anti-*k*		5	
2	*k* anti-*d*	*d*	15	3.5
		k	2	
3	*a* anti-*d*	*d*	14	3.7
		k	0	

* Target cells were P815-X2 mastocytoma cells for experiment 1 and fibroblasts for experiments 2 and 3.

‡ Expressed as Release Index minus Spontaneous Release of target cells alone. This Spontaneous Release was 12 for experiment 1 and 35–42 for experiments 2 and 3. Cell concentrations were adjusted to give ratios of 15 killing cells to 1 target cell for experiment 1 and 9 killing cells to 1 target cell for experiments 2 and 3.

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

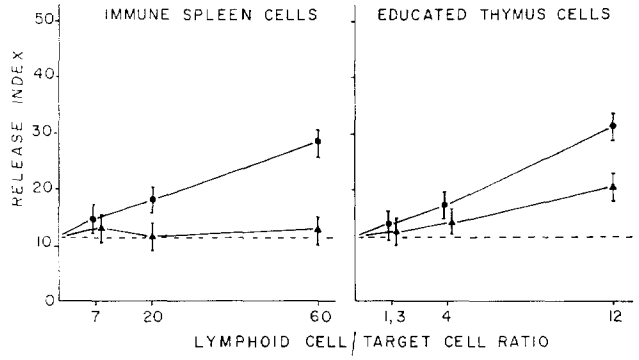

FIG. 3. Residual anti-*d* cytotoxic activity of nonadsorbed *k* anti-*d* immune spleen cells and *a* anti-*d* educated thymus cells after 4 hr of incubation at 37°C in Falcon flasks containing *k* (—●—) or *d* (—▲—) fibroblast monolayers. Cytotoxicity is expressed as Release Index. Dotted line (-----) represents R.I. of target cells alone.

DISCUSSION

Immune cells kill the relevant target cells in vitro. T cells are necessary for the killing, as shown by the effect, in the presence of complement, of antisera directed against T cells, either homologous anti-θ antiserum (15) or our het-

erologous anti–T antiserum. We tried to demonstrate that T cells are sufficient for the killing. For this purpose, we used techniques aiming at enriching killing cell populations in T cells.

One technique was purification of immune spleen cells by filtration through a column of beads coated with anti–mouse Ig antiserum. As shown by four different tests (Table II) this resulted in an almost complete retention of cells that were not T cells, although it is not possible to claim absolute purification. Thus on one hand it is obvious that column-purified immune spleen cells, containing far less B cells than nonpurified immune spleen cells, are not less cytotoxic. On the other hand, one still cannot exclude, at this stage, some participation in the cytotoxic process of a small proportion of contaminating B cells.

A second technique was education of thymus cells. Normal thymus cells were injected into irradiated semiallogeneic recipients. Educated thymus cells were obtained from the spleen of the recipient mice 5 days after injection. To what extent are these educated thymus cells contaminated by B lymphocytes? In the presence of complement, a rabbit anti–T antiserum kills 96% (Table II) and an anti-θ antiserum kills about 90% (our unpublished experiments) of the educated thymus cells. Some of the anti-θ–resistant cells are lymphoid or nonlymphoid cells of recipient origin; it has been shown previously (18) that 5–8% of the "educated thymus cells" are of recipient origin. Some of the anti-θ–resistant cells may be T cells, as it has been shown that not every T cell is sensitive to anti-θ antiserum (31). Finally, it cannot be excluded that a small proportion of the educated thymus cells are in fact B cells, derived from a small quantity of B cells present among the injected normal thymus cells. Thus, on one hand it is obvious that educated thymus cells, containing far less B cells than immune spleen cells, are not less cytotoxic. On the other hand, one still cannot exclude, at this stage, some participation in the cytotoxic process of a small proportion of contaminating B cells.

Therefore, a combination of these techniques was used: educated thymus cells were further purified by filtration through an antiserum-coated column. The resulting column-purified educated thymus cells were usually as cytotoxic or more cytotoxic than nonpurified educated thymus cells. Depletion of B cells (perhaps to the point of complete deprivation?) clearly does not decrease the cytotoxic activity of a population of killing cells. This strongly suggests that B cells are not involved in the killing by these immune cells in vitro. Nonlymphoid cells (macrophages, polymorphonuclears) are very probably not involved either, since column purification is done in conditions which would cause the removal of most of them (32, 33). We would thus conclude that only T cells seem to be involved in the killing of target cells by immune cells in vitro in this experimental system.

Not only this specific killing in vitro by educated T cells, but also the in vivo "education" of normal resting T cells into specifically killing T cells, might not require participation of other cells than T cells. Preliminary experiments have

shown that normal thymus cells, purified by filtration through an anti-mouse Ig antiserum-coated column and then injected into irradiated semiallogeneic recipients, are educated at least as readily as unpurified thymus cells.

Column-purified cells were, in most of the experiments, more cytotoxic than unpurified cells. This might be due to some effect of the columns on the column-purified cells. For instance, anti-Ig antibodies or antigen-antibody complexes detached from the columns might stimulate lymphocytes to kill. However, although this may happen, we think, for the following reasons, that this may not be the main mechanism leading to an increase in cytotoxicity. First, filtration through a column of beads coated with normal, nonimmune serum also led to some increase in cytotoxicity, although this increase was less important than with the antiserum-coated columns. Table II shows that such a column retains a significant proportion of B cells, although the retention is far less important than with the antiserum-coated columns. Second, in some experiments, column-purified educated thymus cells are no more cytotoxic than the initial unpurified cell population. We would be tempted to think that, in these cases, contamination of educated thymus cells by B cells was minimal. It might be that the increase in cytotoxicity observed after column purification is due to removal of B cells. In unpurified immune cell populations, B cells might inhibit to some extent the in vitro cytotoxic activity of T cells.

The fact that, in our system, only T cells seem to be involved in killing has three main implications. The first implication bears on the relationship between the present experimental system and another experimental system used in studies on cell-mediated cytotoxicity in vitro. Normal lymphocytes can kill target cells in vitro, provided that these target cells are coated with antibodies directed against their surface antigens (see reference 12 for review). The question arises whether killing by immune cells and killing by normal cells plus antiserum directed against the target cells might not be two experimental aspects of the same biological phenomenon. Among other arguments, the present findings that only T cells may be involved in one system, whereas B cells, and perhaps only B cells, are involved in the other system (34), strongly suggest that these two systems are indeed distinct. Thus, at least two different pathways for target cell killing by lymphocytes can be demonstrated in vitro. We do not know if the same might also be true in vivo.

The second implication bears on the possibility of cooperation between lymphoid cells in cell-mediated cytotoxicity in vitro. A cooperation between T and B cells has been claimed in a system where rat lymphocytes are first sensitized in vitro on mouse fibroblast monolayers and then tested for cytotoxicity against these fibroblasts. It was found that sensitized thymus cells were able to kill much more efficiently if mixed with fresh spleen cells (35) and that this could be due to a cooperation between sensitized T cells and fresh B cells (35, 36). In our experimental system, unpublished experiments have shown that fresh spleen cells do not increase the cytotoxicity of educated thymus cells.

Furthermore, the experiments described in this paper strongly suggest that T cells alone can kill. Thus, T cell–B cell cooperation does not seem to exist in our system. On the other hand, a T cell–T cell interaction, leading to target cell death, cannot be excluded at present.

Finally, the third implication bears on the problem of the receptor-bearing cells. We demonstrated previously that receptor-bearing cells are involved in killing (17). We show in this paper that T cells are necessary and very probably sufficient for the killing. Thus, receptor-bearing cells seem to be T cells. This is further strengthened by the fact that column-purified educated thymus cells may be specifically adsorbed on the relevant fibroblast monolayers, directly demonstrating the presence of receptor-bearing cells among highly purified T cells.

This experimental system can thus be used for the study of T cell–specific receptors. For instance, present and previous results (17), when put together, lead to the conclusion that at least a majority of T cells is homogeneous as to the specificity of surface receptors, i.e., one T cell seems to bear receptors of only one given specificity. Also, specific adsorption of T cells on fibroblast monolayers seems to be temperature dependent (17), probably because of temperature-dependent events occurring in or on the T cells (unpublished results). As to the nature of T cell–specific receptors, a first indication is given by the nonretention of receptor-bearing killing cells by columns of beads coated with anti–mouse Ig antiserum. Similar results have also been observed when filtering normal spleen cells through these columns and testing the cells for graft-*versus*-host reactivity in vivo. Column-purified cells were not less reactive than unpurified cells.[3] Also, it could be shown in a chicken system that peripheral lymphocytes, after passage through a column coated with purified anti–light chain antibodies, no longer contained any rosette-forming cell against heterologous red cells whereas they had absolutely normal reactivity in a graft-*versus*-host assay.[5] This could mean, either that T cell receptors are not "classical" immunoglobulins, or that their concentration is much lower than the concentration of immunoglobulins at the surface of B cells. Our findings and both these interpretations are consistent with immunofluorescence data detecting the presence of immunoglobulins at the surface of B cells but not at the surface of T cells (37, 38).

SUMMARY

In order to investigate whether only T cells are involved in a cell-mediated cytotoxic system in vitro, we tested the cytotoxicity of immune killing cell populations as deprived as possible of B cells. Educated thymus cells, immune spleen cells purified by filtration through a column of beads coated with anti–mouse Ig antiserum, and finally educated thymus cells further purified by filtration through such a column fully retained their specific cytotoxic activity. This

[5] Crone, M., C. Koch, and M. Simonsen. Manuscript submitted for publication.

very strongly suggests that only T cells are involved in the killing of target cells by allogeneic immune cells in vitro, in this system.

Receptor-bearing cells involved in killing in the present system are thus very probably T cells. This point was further strengthened by the demonstration of specific adsorption, on the relevant monolayers, of each of the three above mentioned killing cell populations.

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