

HETEROGENEITY OF THE EFFECTOR CELLS IN THE
CYTOTOXIC REACTION AGAINST ALLOGENEIC
LYMPHOMA CELLS

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Thymus-derived (T)¹ cells are generally considered to play a central role in cell-mediated cytotoxicity against allografted tumors. Thus, lymphocytes bearing receptors for antigen and responding to allografts are thymus derived (1-4); they are required for the expression of cytotoxicity during the immune response (5, 6); and educated thymus-derived cells can act as killer cells even after purification on antibody-coated columns (2).

Other circulating lymphocytes have also been demonstrated to act as killer cells in allograft reactions (see reference 7 for review). For example, antibody bound to target cells can induce cytotoxicity by activating bone marrow-derived (B) lymphocytes (8, 9).² This antibody-induced cytotoxicity requires the binding of the Fc fragment of the bound immunoglobulin to effector lymphocytes in order for killing to occur (10). While antibody-induced cytotoxicity can be readily demonstrated *in vitro* with sensitized target cells and nonimmune effector cells, few data are available to show that this event actually occurs *in vivo* after specific host sensitization (11-13).

There is other evidence suggesting that more than one cell participates in the allograft response. Thus, the cell(s) required for expressing cytotoxicity in spleens from immune mice vary with time after immunization in their sensitivity to X irradiation (14). Similar differences are noted with respect to cellular morphology, requirements for DNA synthesis, and killing efficiencies (14, 15). However, these effects may also reflect the characteristics of one cell at different stages of maturation rather than different cell types.

We decided to study the host response to an allografted lymphoma from the earliest possible time after host priming through the peak and later stages of the immune response in order to test whether T cells were always required for the mediation of cytotoxicity.

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¹ *Abbreviations used in this paper:* B cells, bone marrow-derived cells; BSS, balanced salt solution; EIS, early immune spleens; GPC, guinea pig complement; [¹²⁵I]UdR, ¹²⁵I-labeled iododeoxyuridine; LIS, late immune spleens; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T cells, thymus-derived cells.

² Britton, S., H. Perlmann, and P. Perlmann. Manuscript in preparation.

Materials and Methods

Animals.—A/Sn, CBA, and (A × CBA)F₁ mice between 2 and 4 mo of age were used. All were age matched and of the same sex for individual experiments.

Tumors.—The YAC (*H-2^d*) Moloney virus-induced ascites leukemia was obtained from the Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden, and was used during its 220th–246th generation in vivo.

Isotopes.—¹²⁵I]Iododeoxyuridine (¹²⁵I]UdR) and ⁵¹Cr]sodium chromate were both obtained from Amersham/Searle Corp., Arlington Heights, Ill.

Immunizations.—Mice were immunized by intraperitoneal inoculation of either spleen cells or 10⁷ live tumor cells. At varying time intervals thereafter, the animals were sacrificed and their spleens or mesenteric lymph nodes excised using sterile technique. A cell suspension was made in medium as described by Mishell and Dutton (16) and the number of viable cells was determined by trypan blue staining.

Pretreatment of Cells.—Spleen cells were pretreated with antitheta or normal (AKR) serum and complement. Antitheta serum was prepared as described by Greaves and Möller (19) and different batches of such sera were obtained by bleeding mice at different times. 50 × 10⁶ spleen cells were incubated with 0.5 ml of antitheta or AKR serum at a 1:1 or 1:3 dilution for 15 min at 37°C. The cells were then washed, the pellet resuspended in 1.0 ml of guinea pig complement (GPC) that was preabsorbed with agarose (18) and mouse spleen and liver cells, and used at a 1:1 or 1:3 dilution. After rocking the suspension for 45 min at 37°C, the cells were washed twice and the number of viable cells determined before use. In general, such treatments rendered between 20 and 38% spleen cells, 47 and 62% lymph node cells, and 94 and 97% thymus cells nonviable when CBA mice were used.

In order to remove phagocytic cells, some suspensions were pretreated with carbonyl iron powder according to the method of Lundgren et al. (19). After 30 min incubation with the iron powder at 37°C with rocking, the iron was extracted in several steps with the aid of a magnet and the cells were then washed twice before further experimental treatments.

Labeling of Target Cells.—Peritoneal YAC cells were removed under sterile conditions from (A × CBA)F₁ tumor-bearing mice between 6 and 8 days after intraperitoneal inoculation of 10⁷ cells. The cells were washed once and placed in a Falcon flask (no. 3024 Falcon Plastics, Oxnard, Calif.) at a concentration of 1.0–1.5 × 10⁶ cells/ml. The medium containing the cells was a modified minimal essential medium with supplements (20) and also included 20% fetal bovine serum. To this suspension, ¹²⁵I]UdR was added at a concentration of 0.07 μCi/ml and the flask was rocked for 4 h at 37°C. The cells were then washed twice at low-speed centrifugation and resuspended to the appropriate concentration in balanced salt solution (BSS). In some experiments, cells were doubly labeled with ⁵¹Cr]sodium chromate and ¹²⁵I]UdR. In order to do this, the ¹²⁵I]UdR-labeled cells were suspended in 2 ml of medium with ⁵¹Cr using 100 μCi Cr/50 × 10⁶ cells. After 45 min incubation at 37°C, the cells were washed six times before use.

Cytotoxic Assays.—The method used here follows the original description of the method for keeping mouse spleen cells alive in vitro (16) and is similar to that described by Canty and Wunderlich (21). Spleen cells were suspended in medium with 10% heat-inactivated fetal calf serum and 1 ml of the suspension was placed in 35-mm plastic Petri dishes. 0.1 ml of the tumor cell suspension (usually 10⁵ cells) was then added to these dishes and they were placed in a sealed box with a gas mixture of nitrogen (83%), oxygen (7%), and carbon dioxide (10%) and rocked at 37°C. Unless otherwise indicated, the reactions were run for 16 h. The cell suspensions were then removed from the individual Petri dishes (three dishes/group) with the aid of a rubber policeman. This suspension was centrifuged and the entire supernatant removed carefully with a disposable pipette and saved (first supernatant). The pellet was resuspended on a Vortex mixer and 0.3 ml of a 0.25% trypsin solution added. After 30 min incubation at 37°C, 0.7 ml of cold BSS was added and the tube centrifuged. One-half of the supernatant was re-

moved (second supernatant) and these two supernatants as well as the tube containing the cell pellet were placed in a well-type gamma counter and the percent release of ^{125}I was calculated using the formula (bk = background):

$$\% \text{ } ^{125}\text{I} \text{ release} = \frac{\text{cpm 1st supernatant} - \text{bk} + 2 (\text{cpm 2nd supernatant} - \text{bk})}{\text{cpm 1st} + 2 \text{nd supernatant} + \text{pellet} - 3 (\text{bk})} \times 100 .$$

In some experiments the release of isotope was expressed as a cytotoxic index:

Cytotoxic index =

$$\frac{\% \text{ release } ^{125}\text{I} \text{ with immune cells} - \% \text{ release } ^{125}\text{I} \text{ with nonimmune cells}}{\% \text{ release } ^{125}\text{I} \text{ with immune cells}} \times 100 .$$

Local Hemolysis in Gel Assay.—The technique originally described by Jerne and Nordin and modified by Mishell and Dutton (16) was used. In each experimental group, cells from three dishes were pooled, spun once, and resuspended in cold BSS to a concentration suitable for the plaquing assay. The cells were mixed with the appropriate erythrocytes in agarose and the mixture poured onto glass slides. Duplicate slides were made for each group. For detection of plaque-forming cells (PFC), the slides were incubated at 37°C for 3 h in the presence of GPC diluted 1:20 in BSS.

Antigens.—Sheep erythrocytes (SRBC) were prepared and used for the in vitro experiments as previously described (22). 10^7 SRBC in $30 \mu\text{l}$ was the immunizing dose and this was mixed with spleen cell suspensions containing 10×10^6 cells in 1 ml in individual Petri dishes. The cultures were fed each day with a nutritional cocktail (23). In these experiments the spleen cells had been pretreated with antitheta or normal mouse serum and complement.

Antibody Titrations.—Cytotoxic antibodies were determined by mixing equal volumes (0.05 ml) of antiserum in serial dilutions with YAC target cells (5×10^5) and incubating this mixture at 37°C for 30 min. The cells were then washed once and 0.1 ml of 1:3-diluted GPC absorbed with agarose (18) was added and the mixture was incubated at 37°C for another 45 min. After this treatment, cell death was determined either by trypan blue dye exclusion or ^{51}Cr isotope release.

RESULTS

Characteristics of the Assay System.—In these experiments, target cells were labeled with ^{125}I UdR and the percent release of ^{125}I from the tumor cells was used to estimate cell death. This method has been shown to be a reliable indicator of cell survival (24) and was used because the in vitro labeling procedure required a minimum of handling of the target cells. When ^{125}I UdR was added to a rocking suspension of YAC cells, 65% of the label was precipitable with 10% trichloroacetic acid after 24 h (Fig. 1). However, sufficient label was incorporated into the cells (2000 cpm/ 10^5 cells) after 3–4 h to allow their use as target cells in the assay.

Release of radiolabel from cells incubated alone or with normal spleen cells was between 15 and 25% after 16 h. Target cells incubated with specifically immune cells released isotope in proportion to the effector to target cell ratio (Figs. 2 and 8). Further, when YAC cells were doubly labeled with ^{125}I and ^{51}Cr , the release of both isotopes from target cells in the presence of immune cells was similar (Fig. 2).

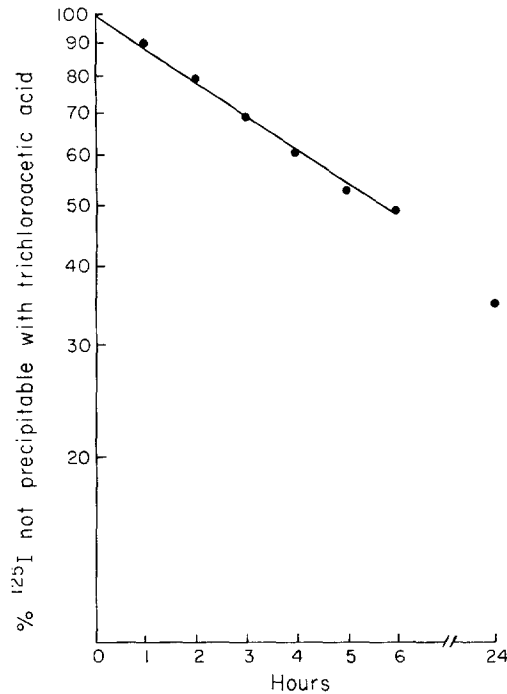


FIG. 1. Incorporation of [^{125}I]UdR into YAC cells. Points represent the percent of total ^{125}I activity not precipitable in 10% trichloroacetic acid after addition of [^{125}I]UdR to a cultured suspension of YAC cells.

A few precautions were found to be important when using this label in the assay. Firstly, it was necessary to trypsinize the cells after the incubation period of the test to assist in the release of some of the label from dead cells, which probably had not disintegrated sufficiently to liberate their nuclear contents. Secondly, the cells could not be labeled with a high concentration of ^{125}I because of the radiotoxic properties of this isotope (25).

Characteristics and Specificity of the Immune Response to the Allogeneic Lymphoma YAC.—CBA mice were divided into groups of three and inoculated intraperitoneally with 10^7 YAC cells on different days. Subsequently, the groups were all sacrificed on the same day, the spleens pooled within each group, and their cytotoxic activity evaluated from three replicate cultures per group. In Fig. 3 it can be seen that lymphocytes from the immunized *H-2* allogeneic mice developed a cytotoxic effect which peaked between 7 and 14 days after priming. After this time there was a gradual decline in the degree of cytotoxicity reaching a minimum at day 32. Immunity was also detected with cells derived from the mesenteric lymph nodes (Table I).

The specificity of this response was tested with respect to *H-2*- and tumor-specific immunity. Specific *H-2* immunity was demonstrated since CBA

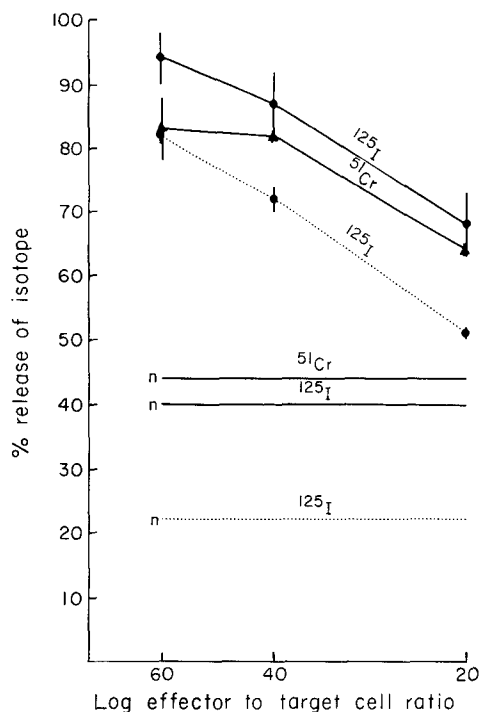


FIG. 2. Percent ^{125}I and ^{51}Cr release from singly and doubly labeled YAC cells. Percent release of $^{125}\text{I} \pm \text{SEM}$ from YAC cells in the presence of nonimmune (n) or immune CBA spleen cells. ----, isotope release from YAC cells labeled with [^{125}I]UdR; —, isotope release from YAC cells labeled with [^{125}I]UdR and ^{51}Cr . ^{51}Cr and ^{125}I release was calculated using the formula found in the Materials and Methods section.

($H-2^k$) mice sensitized with A/Sn ($H-2^a$) spleen cells killed YAC ($H-2^a$) target cells while A/Sn mice sensitized with CBA spleen cells did not (Table II). To test for tumor-specific immunity, CBA mice were sensitized with YAC or YBB cells, the latter being a Moloney virus-induced lymphoma that originated in a CBA mouse. Here the response was also specific since after 3 days the spleens from mice sensitized with YAC showed a strong cytotoxic effect against YAC target cells while those primed with YBB showed only a very weak effect (Table II).

Effect of Antitheta Serum on Cell-Mediated Cytotoxicity.—Mice were inoculated with 10^7 YAC cells intraperitoneally. At varying time intervals thereafter, groups containing three mice each were sacrificed; their spleens were pooled and then treated with samples from a pool of antitheta or normal (AKR) mouse serum and GPC. After treatment, equivalent numbers of viable cells were added to the target cells to compare the degree of cytotoxicity between the two groups. It was found that spleen cells taken from mice before the 6th day

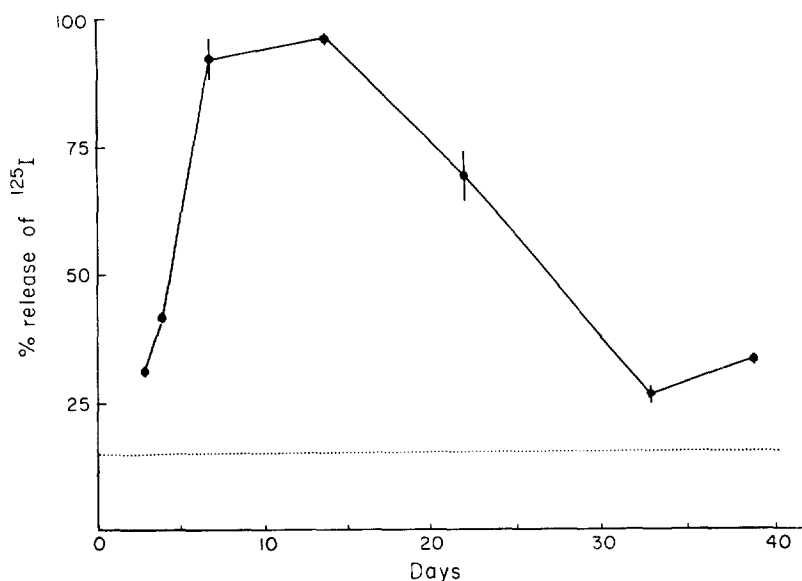


FIG. 3. Cytotoxic effect of CBA spleen cells. Percent release of $^{125}\text{I} \pm \text{SEM}$ from YAC cells in the presence of immune CBA spleen cells taken at various time intervals after inoculation with YAC cells. Dashed line represents isotope release in the presence of nonimmune CBA cells. Effector to target cell ratio, 100:1.

TABLE I
Cytotoxic Effect of Immune Spleen and Mesenteric Lymph Node Cells

Effector cell	Cell source	^{125}I release
		%
None	—	10.2
Nonimmune	Spleen	15.3
Nonimmune	Mesenteric lymph node	17.0
Immune	Spleen	77.0
Immune	Mesenteric lymph node	54.6

Animals primed with 10^7 YAC cells 6 days before spleen and lymph nodes were removed for testing.

Effector to target cell ratio, 100:1.

after priming (early immune spleens or EIS) were not sensitive to antitheta serum treatment because there was no reduction in the cytotoxicity observed (Fig. 4). However, after this time and during the peak and later stages of the response, there was suppression of cytotoxicity in these spleens (late immune spleens or LIS) after antitheta serum treatment.

Since the spontaneous release of isotope from the target cells varied considerably at different times of assay, we also performed experiments where groups of

TABLE II
Specificity of Cytotoxic Effect

Donor cells	Host	Days after host priming test performed	Effector to target cell ratio	Release of ¹²⁵ I with nonimmune cells	Release of ¹²⁵ I with immune cells
				%	%
A/Sn	CBA	7	100:1	10.7	40.3
CBA	A	7	100:1	10.7	11.4
YAC	CBA	4	60:1	10.7	48.7
			30:1	11.6	33.3
YAC	A/Sn	4	60:1	9.4	8.8
			30:1	10.5	9.2
YAC	CBA	3	100:1	19.9	46.0
YBB	CBA	3	100:1	19.9	26.8

Mice were primed with 10⁷ live spleen or tumor cells.

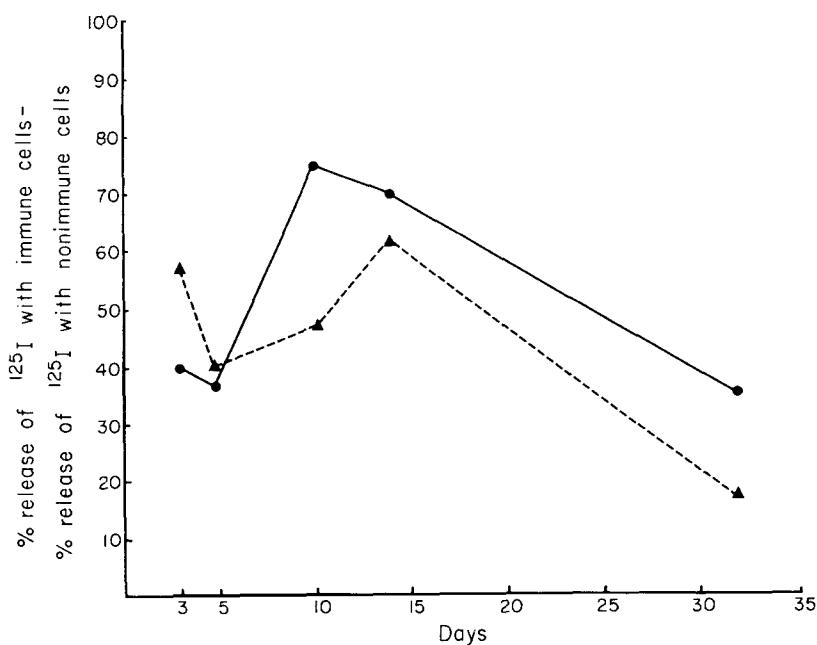


FIG. 4. Cytotoxic effect of antitheta serum-treated spleen cells. Percent release of ¹²⁵I from YAC cells in the presence of immune CBA spleen cells — percent release of ¹²⁵I in the presence of nonimmune CBA spleen cells. Animals were primed with YAC cells and tested at various time intervals. —, immune spleen cells pretreated with AKR serum and GPC; or ----, antitheta serum "A" and GPC. Effector to target cell ratio, 100:1.

mice were inoculated with tumor cells on different days and then tested all on the same day. Similar results were observed in this case; i.e. killer cells from EIS were not theta sensitive nor was the presence of theta-positive cells necessary for the expression of cytotoxicity, while the mediation of cytotoxicity in LIS required, at the least, the presence of a theta-positive cell (Fig. 5). Cytotoxicity in LIS was always inhibited by the antitheta serum treatments, but the amount of suppression varied and was never complete as others have reported (6). This may have been due to either the persistence of some T cells

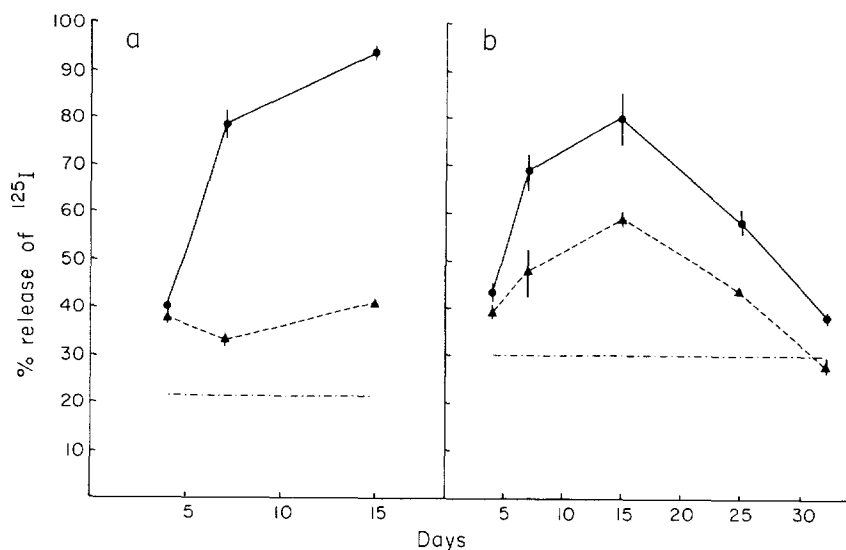


FIG. 5. Cytotoxic effect of antitheta serum-treated spleen cells. Percent release of $^{125}\text{I} \pm$ SEM from YAC cells in the presence of immune CBA spleen cells. Mice were primed at different time intervals with YAC cells and their spleens were tested on the same day. —, CBA immune spleens pretreated with AKR serum and GPC; ----, CBA immune spleens pretreated with antitheta serum "H" and GPC in panel a, and "E" in panel b; - - - - -, percent release of ^{125}I in the presence of nonimmune spleen cells. Effector to target cell ratio, 100:1.

after the treatments (26), or residual activity of a postulated antitheta serum-resistant cell population existing in the EIS.

The rate of radioiodide release from YAC cells incubated with day 5 immune spleens (Fig. 6) was rapid, with the peak rate occurring during the first 6 h of the test. In this experiment, as well as in some of the others we performed (e.g., Fig. 4), an enrichment or increase in cytotoxicity was seen after antitheta serum pretreatment of early immune cells.

To test the efficiency of the antitheta sera used in the above experiments in removing those T cells required for "helper" activity, we tested the ability of serum-treated spleen cells to respond to heterologous erythrocytes *in vitro* where the number of PFC was measured 4 days after the addition of SRBC.

We found that these antitheta sera were able to inhibit such a response by 60-95% (Table III) similarly to what others have observed (27).

Effect of Iron Treatments of Immune Spleen Cells.—Since the cell participating in EIS responding to YAC was not sensitive to antitheta serum, we wanted to

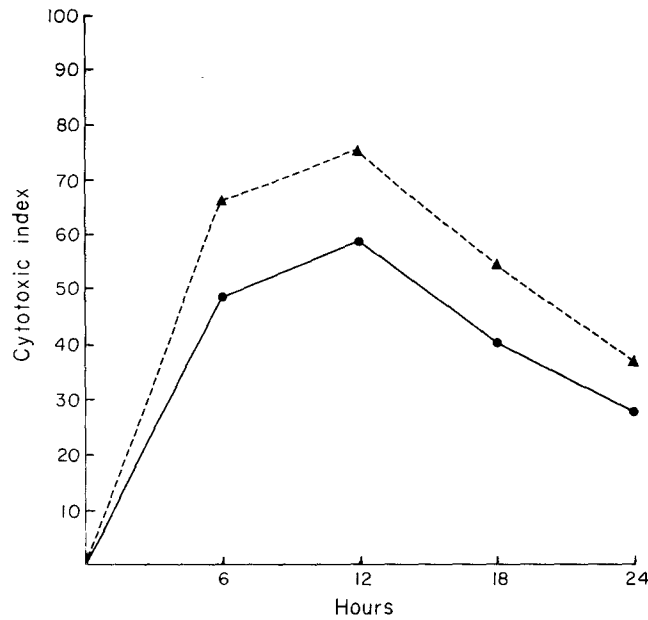


FIG. 6. Rate of ¹²⁵I release from YAC cells in the presence of immune CBA spleen cells. Index of ¹²⁵I release from YAC cells in the presence of immune CBA spleen cells taken from mice primed 5 days previously with YAC cells. Spleen cells were pretreated with: —, AKR serum and GPC; or ----, antitheta serum "B" and GPC. Isotope release was determined at 6-h intervals after the start of the assay. Effector to target cell ratio, 100:1.

TABLE III

The Effect of Treatment of (A × CBA)F₁ Spleen Cells with Antitheta or Normal Mouse (NMS) Sera and GPC on Their Ability to Make PFC against SRBC. Test after 4 days Culture

Exp.	Spleen cell pretreatment	Dilution of antitheta sera and GPC	No. of viable cells recovered (× 10 ⁶)	Total no. of PFC — background/culture	Reduction in PFC after antitheta pretreatment %
1	NMS	1/3	7.6	388	
	Antitheta "D"	1/3	7.4	111	72
2	NMS	1/1	5.8	545	
	Antitheta "B"	1/1	6.4	217	60
3	NMS	1/3	6.6	422	
	Antitheta "A"	1/3	3.8	23	95
	Antitheta "H"	1/3	4.6	32	92

determine if nonlymphoid cells were involved in mediating cytotoxicity at this time. Animals primed 3 days before testing had their spleens removed and a portion of the cells were pretreated with carbonyl iron powder in order to remove adherent cells (28). The cells were then assayed for their cytotoxicity against YAC and compared with equivalent numbers of nontreated spleen cells. In Fig. 7, it can be seen that such treatments did not reduce the amount

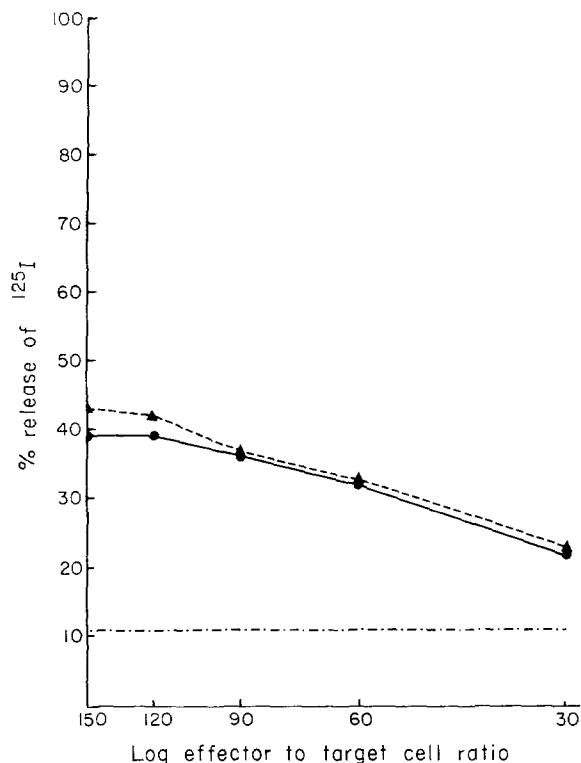


FIG. 7. Cytotoxic effect of carbonyl iron-treated immune CBA spleen cells. Percent release of ^{125}I from YAC cells in the presence of CBA spleen cells taken from mice primed 3 days previously with YAC cells. Spleen cells were pretreated with carbonyl iron powder, ----; or untreated, —., isotope release in the presence of nonimmune cells.

of isotope release from target cells, and thus phagocytosing or adherent cells do not appear to participate in this stage of the immune response.

Effect of Antitarget Cell Immune Sera on Cytotoxicity Caused by Normal and Immune Spleen Cells.—When normal spleen cells, purified by treatment with carbonyl iron, were added to YAC cells in the presence of sera from CBA mice in the process of tumor rejection or a hyperimmune A.CA anti-*H-2^a* serum, cytotoxicity was induced at serum dilutions as low as 10^5 . This antibody-induced cytotoxicity has been described previously (see references 7 and 8 for

review) and will be reported in this series later (manuscript in preparation). In these experiments, we wanted to study whether spleen cell populations that varied in their sensitivity to antitheta serum treatments also responded differently in the presence of antitarget cell antisera with regard to expression of cytotoxicity.

CBA mice were immunized with YAC cells at different time intervals and sacrificed on the same day. Their spleen cells were pretreated with carbonyl iron to remove adherent cells and then added to dishes containing target cells. At the same time an A.CA anti-A or normal A.CA heat-inactivated serum was added to the cultures at varying dilutions. In Fig. 8, results of such experiments are presented. The tumor cells released 20% of their label when incubated with normal CBA spleen cells. When the target cells were mixed with day 3 or day

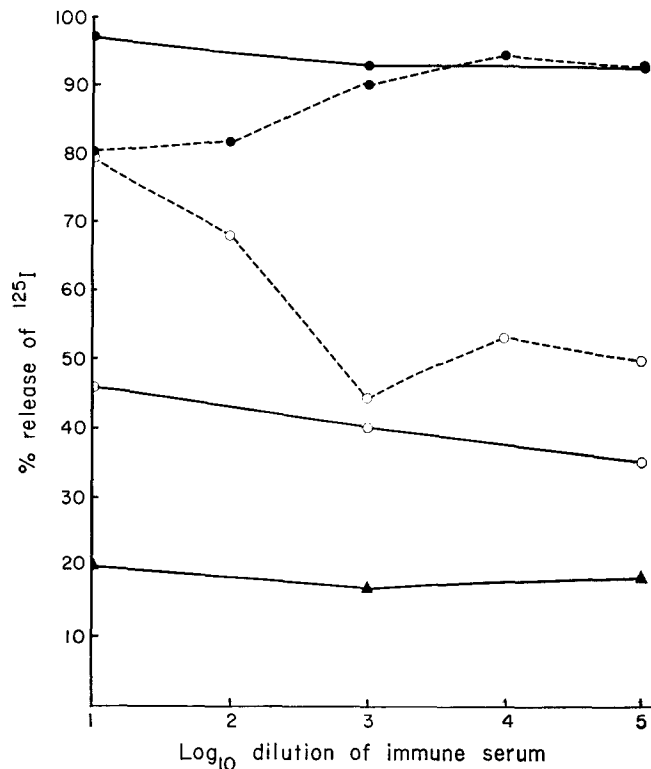


FIG. 8. Effect of anti-target cell immune serum on the cytotoxic effect of immune CBA spleen cells. Percent release of ^{125}I from YAC cells in the presence of CBA spleen cells taken from mice primed 3 (○) or 9 (●) days previously with YAC cells. ▲, nonimmune mice; ----, A. CA anti-A/Sn serum; or —, normal A. CA serum added to the cultures at various dilutions. Immune serum was obtained from A. CA mice after receiving four weekly injections of A/Sn spleen cells. Effector to target cell ratio, 100:1.

9 immune cells, approximately 40 and 95% of the isotope was released, respectively, in the presence of normal mouse serum. When immune mouse serum was added to dishes containing day 3 immune cells, cytotoxicity was greatly increased compared with those groups with normal mouse serum. However, when the same antiserum at the same dilutions was added to dishes with day 9 immune cells (Fig. 8) or day 10 or day 21 immune cells (data not shown) the release of isotope was reduced. Thus, in the presence of immune serum, there was a differential cytotoxic effect of spleen cell populations, depending on the time after priming that they were tested.

Characteristics of the Killing Efficiency of Immune Spleen Cells.—When varying numbers of effector cells were added to a standard number of target cells, the degree of isotope release increased linearly with the log of the effector to target cell ratio. A plot of the data obtained from testing spleens taken at two different time intervals after priming showed that there were more effector cells on day 7 of the immune response compared with day 3 (Fig. 9). However, as the effector to target cell ratio was increased, target cell lysis in the presence of the day 7 immune cells increased at a greater rate than in the presence of cells taken 3 days after priming, suggesting a different efficiency or character of

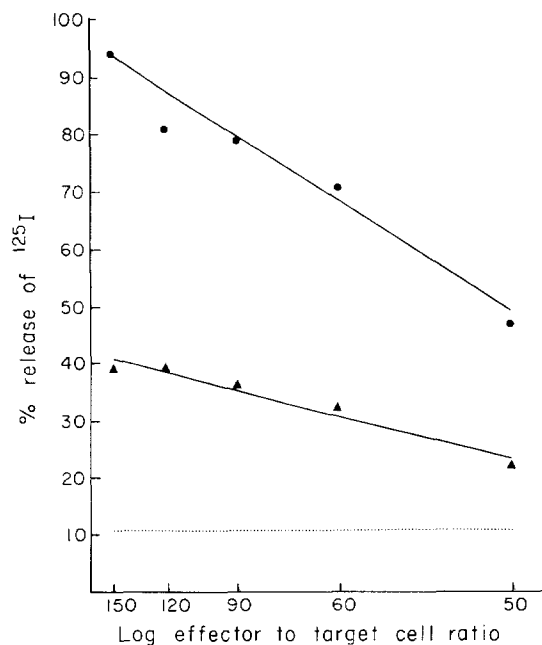


FIG. 9. Cytotoxic effect of day 3 and day 7 immune CBA spleen cells. Percent release of ^{125}I from YAC cells in the presence of CBA spleen cells taken from mice primed 7 days (●) or 3 days (▲) previously with YAC cells. ----, release of isotope in the presence of nonimmune spleen cells.

cell killing (15). This finding is consistent with either a different effector cell operating at these time intervals, or the same cell but at different stages of maturation.

DISCUSSION

Before discussing the results of the host response we would like to comment on the assay system used here. This is important because the amount of cytotoxicity observed during the early phase of the immune response in the presence of spleen cells was small and required a sensitive method for its demonstration. In our hands, ^{51}Cr labeling involved considerable handling of our target cells which resulted in a high spontaneous isotope release at the end of the assay (after 16 h). Therefore, we selected [^{125}I]UdR as the indicator of cell death since it offered advantages such as (a) ease of labeling cells under usual tissue culture conditions, (b) minimal handling after labeling, and (c) absence of leakage from viable cells. However, since spontaneous isotope release is a function of the target cell's ability to survive the conditions of the assay system used, the suitability of a particular labeling method will vary with different test systems.

Others have shown that ^{125}I release from [^{125}I]UdR-labeled cells is an accurate indicator of cell death (24). In these experiments, we found that this label was released from target cells in proportion to the number of immune cells present and similar to ^{51}Cr release when the cells were doubly labeled. After 16 h incubation of target cells with immune spleen cells taken from mice primed 3-4 days before testing, 40-50% of the total label was found in the supernatant and by day 7 this figure rose to greater than 90%, while background release of ^{125}I in the presence of nonimmune spleen cells was usually about 20%. Further, the uptake of [^{125}I]UdR by the cultured tumor cell suspensions during the labeling procedure was rapid which allowed the usage of the target cells on the labeling day.

Non-thymus-derived lymphoid cells have been demonstrated to act as killer cells in cell-mediated cytotoxic reactions when the target cell has been sensitized with antitarget antibody (8, 9, 29). The fact that the target cell can be lysed in the presence of purified spleen preparations when thymus-derived and phagocytosing cells have been removed indicates that bursal equivalent of B cells are capable of mediating this effect.²

Reports of experiments done *in vivo* have indicated that serum antibody may indeed play a role in graft rejection (11-13). Whether this is due to a cell-mediated mechanism, however, is still not clear.

In this report we have presented evidence that is compatible with a cell-mediated cytotoxic response that may involve non-thymus-derived lymphocytes as the final effector cells in graft destruction. Our data show that the cytotoxic effect of spleen cells taken soon after host priming was not sensitive to antitheta sera and complement. However, if spleens taken from mice during the

peak and later stages of the response were pretreated with antitheta sera, suppression of cytotoxicity occurred. This indicates that a cell lacking or low in theta antigenic content is the early killer cell, with a second theta-positive cell participating at a later stage of the immune response and required to attain peak cytotoxicity. The second (theta-positive) cell may be a component of the recirculating lymphocyte pool migrating from an extra splenic site to the spleen (30) or be generated from the thymus-derived antigen recognizing cell population residing in the spleen before sensitization (31). Whether this second cell is the actual killer cell, though, cannot be determined from these experiments. Since spleen cell cytotoxicity was decreased but not completely eliminated during the peak response by the antitheta sera treatments, this residual killing may indicate that these postulated non-theta-positive cells persist throughout the immune response, although we do not exclude the possibility that these treatments leave some surviving T cells as others have found (23, 26, 27). If the antitheta-resistant cell is a B cell participating in antibody-induced cytotoxicity, then the relatively weak cytotoxic effect exerted by these cells may indicate that some YAC cells are resistant to cytolysis by this mechanism.

The fact that the antitheta sera suppressed the primary *in vitro* PFC response to SRBC as well as the cytotoxic effect of spleen cells taken from mice during the peak and later stages of the immune response indicates that such treatments were effective in inhibiting T cell function. Further, although the early proliferating T cell responding to allogeneic tissue in our system may be a large pyrinophilic blast cell as others have found (1, 14), it is unlikely that this cell is resistant to antitheta sera since it has been demonstrated that PHA-stimulated blast cells are susceptible to such treatment (32). However, the theta-resistant effector cell seen in the EIS could still be a T cell at some stage of maturation or differentiation that is resistant to the antisera treatment or one that does not participate in helper cell activity.

When spleen cells from day 3 immune mice were treated with carbonyl iron powder to remove adherent cells (28), there was no decrease in the cytotoxic effect to YAC. Thus, while macrophages have been shown to participate in cell-mediated immune responses (33, 34), it is unlikely that this cell type was the theta-resistant killer cell in these experiments.

This host response was also demonstrated to be specific for the *H-2* antigens of the *H-2^a* allele since *H-2^a* anti-*H-2^k* spleen cells did not kill YAC. Tumor-specific immunity due to tumor-specific antigens induced by Moloney virus was probably minimal with this immunizing regimen since CBA anti-YBB (a CBA Moloney virus-induced leukemia) cells exerted only slight cytotoxicity against YAC cells.

A second difference between these early and later immune spleen cell populations was observed in the presence of immune serum. Such serum was capable of inducing cytotoxicity against YAC cells using nonimmune, iron-purified, spleen cell populations as effector cells. However, in the presence of immune

spleen cell populations, the same serum caused an enhanced cytotoxicity when cells were taken from EIS and inhibited cells derived from LIS. During the early phase of the immune response it is probable that this serum induced unsensitized spleen cells into cytotoxicity and thus augmented the already present cytotoxic effect. The inhibition of the peak phase of cytotoxicity by the same serum could have been a result of direct action on T cells and/or inhibition of B cell killing by high antiserum titers.

It has been demonstrated that sera from tumor-bearing animals taken at various stages of tumor development and regression possess characteristics that enable them to inhibit or promote immune cell function (35). According to the results obtained in this investigation, these "blocking" and "unblocking" sera may vary in their function depending on the stage of the immune response the effector cells are tested at.

A third difference in these two types of immune spleen populations was noted with regard to the slopes of the killing curves when the effector to target cell ratio was varied. Thus, target cell lysis in the presence of day 7 immune cells increased at a greater rate than in the presence of cells tested 3 days after priming. Such findings have been reported previously by Canty et al. (15) who suggested that this reflects a difference in the activity of the immune cell. We also feel that this can indicate either two different cell types as killer cells in the immune response or the same cell at a different stage of maturation. This may also represent an increase in receptor affinity on the killer cell which could occur during the development of the host immune response.

It is not known what type of grafted cell can induce a response in the host that would favor antibody-induced killing. The susceptibility of the target cell to lysis in this kind of cytotoxicity may be related to the expression and distribution of the relevant antigens on the cell membrane, which has been shown to be an important factor with respect to complement-dependent lysis and enhancement of tumor growth (36-38). Finally, one should not expect to find this B cell effector mechanism operational during the course of the host's response unless it can be shown that the tumor cell is sensitive to antibody-induced killing using normal lymphoid cells and immune sera obtained from the appropriate host during the time of tumor rejection.

SUMMARY

The cytotoxic effect of spleen cells from *H-2* allogeneic mice was tested in vitro against an A strain leukemia (YAC) labeled with [¹²⁵I]iododeoxyuridine. After the mice were primed with tumor cells, significant and specific *H-2* immunity was detected on day 3 and peak cytotoxicity was observed between 7 and 14 days after priming. Two effector cells appear to be involved in the host response, because spleens taken from mice soon after priming were not sensitive to antitheta sera and complement while those taken during the peak stages of the response showed a marked reduction in cytotoxicity after treat-

ment. Macrophages were not involved, since removal of these cells by the carbonyl iron method did not result in any reduction in cytotoxicity. Immune serum that was capable of inducing cell-mediated cytotoxicity in normal spleen cell populations also augmented cytotoxicity of spleen cells taken from mice primed 3 days previously. However, when spleen cells were taken from mice during the peak phase of the immune response, the same serum at the same dilutions inhibited the preexisting cytotoxicity. A difference was also detected in the killing efficiencies between early and late immune cells.

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