

ESCAPE FROM ISOANTISERUM INHIBITION OF LYMPHOCYTE-MEDIATED CYTOTOXICITY*

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Lymphocytes derived from animals immunized against alloantigens exhibit in vitro cytotoxicity for target cells carrying the respective isoantigen (1). This cytotoxic activity is mediated by theta-bearing (thymus-derived) lymphocytes (T cells)¹ (2, 3). The accumulated evidence indicates that the cellular immune response to an isoantigen or tumor cell exists concomitantly with isoantibodies secreted by bone marrow- or bursa-derived cells (B cells) that interfere with the in vitro cytotoxic activity of immune T cells (1, 4). It is now possible to quantitate the cytotoxic activity of sensitized lymphocytes through the use of ⁵¹Cr release from labeled target cells, a procedure developed by Brunner et al. (1) and Holm and Perlman (5).

Using what we believe to be a more sensitive adaptation of the Brunner assay system (6), we have found that isoantiserum inhibition of lymphocyte-mediated cytotoxicity (LMC) is not static. The isoantiserum blocking of LMC decreases significantly during our 4-h assay. This decrease in blocking activity, which is evidenced by an increase of 40–60% in the specific ⁵¹Cr released, occurs in the presence of excess antiserum. These results are best understood in terms of antigenic modulation on the target cell (7).

Materials and Methods

Cell Lines and Culture Conditions.—The P-815-X2 mastocytoma (8) was obtained from Dr. T. Brunner and was maintained by weekly transfer of ascitic fluid in DBA/2J mice (Jackson Laboratory, Bar Harbor, Maine). Every 6–8 wk aseptically aspirated ascitic fluid served as inoculum for a tissue culture cell line. The in vitro culture cells were maintained in Dulbecco's modified Eagle's medium with 10% calf serum (CS) (9), at 37°C, in a humidified 15% CO₂-85% air incubator. The cell concentration was maintained at $1-3 \times 10^5$ cells/ml. Under these conditions a generation time of 10 h was routinely recorded (Fig. 1).

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¹ *Abbreviations used in this paper:* B cells, bursa- or bone marrow-derived cells; CS, calf serum; LMC, lymphocyte-mediated cytotoxicity; NMS, normal mouse serum; RPMI, RPMI 1640 medium plus 10% or 20% calf serum; IS, isoantiserum; T cells, thymus-derived cells.

Preparation of Sensitized Spleen Cells and Antimastocytoma Isoantisera (IS).—Mastocytoma cells for immunization were collected by aspirating ascitic fluid from DBA/2J mice. The cells were washed by centrifugation and counted in Alsever's solution (10). For primary immunization, 3×10^7 cells (1) were injected intraperitoneally with 10^9 pertussis organisms into 6–8-wk old C57/BL6 male mice (Jackson Laboratory). 9–12 days after the primary immunization, spleens removed from recipients were minced in RPMI medium (Associated Biomedic Systems, Inc., Buffalo, N. Y.) containing 10% CS and homogenized with a loosely fitting Teflon homogenizer. The spleen cell suspensions were passed through a stainless steel screen (no. 200 mesh) and centrifuged at 350 g for 10 min. The pellet was suspended in the same medium, and lymphocytes were purified by Ficoll-Hypaque centrifugation, as described by Thorsby and Bratlie (11). The gradient purified lymphocytes were then collected by centrifugation, washed with fresh media, and used in the cytotoxic assay. Spleen cells prepared in this manner were 85–99% viable as determined by trypan blue exclusion (12). All procedures were carried out at room temperature.

Isoantiserum was prepared in C57/BL6 mice by injecting 3×10^7 mastocytoma cells 14 days after the primary injection and weekly thereafter for a total of five doses. 7 days after the final booster, the mice were bled from the axillary area. Sera were pooled and decomplexed by incubating at 56°C for 45 min. Sera prepared in this manner had hemagglutination titers of 1,056, as determined by the method of Stimpfling (13).

Labeling of Mastocytoma Cells with ^{51}Cr .—Aliquots of the stock cultures were routinely diluted to 1×10^4 cells/ml and allowed to grow 48 h to assure the use of healthy exponentially growing cells in the experiment (Fig. 1). Cells were collected by centrifugation at 350 g for 3 min. The pellets, containing approximately 5×10^6 cells, were suspended in 1.0 ml of RPMI medium with 10% CS containing 0.25 μg ionic ^{51}Cr (specific activity 300 ~ 400 $\mu\text{Ci}/\mu\text{g}$) (Rachromate injection; Abbott Laboratories, North Chicago, Ill.). The cell suspension was lightly gassed with CO_2 before a 45-min incubation at 37°C.

After incubation, the entire reaction mixture was layered on RPMI medium containing 20% CS and centrifuged at 200 g for 3 min. Sedimentation of the cells through 20% CS at slow speed was repeated five times. This procedure proved to be the most efficient means of getting the background to 5–10% of the total releasable counts. It consistently produced a specific activity of $1.2\text{--}2.0 \times 10^4$ cpm/ 10^5 cells with a viability near 100%, as determined by trypan blue exclusion (12). (Unless specified, all manipulations were carried out at room temperature.)

Cytotoxic Assay.—Spleen and ^{51}Cr -labeled mastocytoma cells were combined in equal proportions. 1 ml of the cell suspension containing 10^5 mastocytoma cells and $0.1\text{--}1 \times 10^7$ spleen cells was incubated at 37°C, in a humidified incubator, in 35-mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) on a rocking platform (6 cycles/min) (4).

For measuring the cytotoxic effect, chromium released into the culture medium was monitored as follows. At each sample time, the entire reaction mixture from one Petri dish was transferred to a centrifuge tube. The cells were pelleted at 450 g for 10 min. A measured aliquot of the supernatant was transferred to counting tubes, and the radioactivity was determined by a Nuclear-Chicago gamma scintillation counter (Nuclear-Chicago, Des Plaines, Ill.). The total releasable count of the reaction mixture was determined by adding 2.0 ml of distilled water to a pellet of a known volume of the reaction mixture removed at the start of the assay. This procedure released 80–90% of the bound ^{51}Cr . The percent specific ^{51}Cr release was calculated according to the method of Brunner et al. (1).

RESULTS

Optimal Conditions for Maximal Lymphocyte-Mediated Cytotoxicity.—In vitro assay methods using ^{51}Cr release from labeled target cells have proved to be very useful in measuring cytotoxic activity of sensitized lymphocytes

(5, 6). In order to study the effect of isoantibodies on the cytotoxic activity of sensitized lymphocytes, we modified the previously described method (1) to improve the sensitivity and reproducibility of the assay system.

First, conditions were sought that gave maximal ^{51}Cr uptake with the least

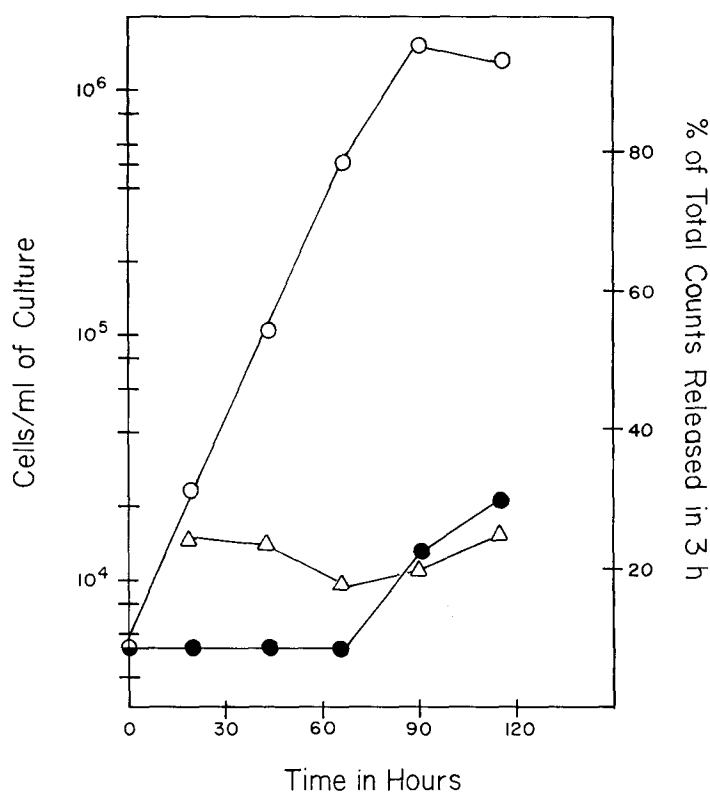


FIG. 1. Uptake and spontaneous release of ^{51}Cr during the growth cycle of mastocytoma. Mastocytoma cells were inoculated to a density of 5×10^3 cells/ml in Dulbecco's modified Eagle's medium at the start of the experiments. At the times indicated by points on the graph, samples were withdrawn. The cell number per milliliter was determined using a hemacytometer; labeling conditions and the determination of the percent spontaneously released counts are described under Materials and Methods. Cell density (○); ^{51}Cr uptake/ 10^5 cells (△); degree of spontaneous ^{51}Cr release in 3 h (●).

amount of spontaneous cell lysis. A culture of mastocytoma cells was started at low density (i.e. 5×10^3 cells/ml) for establishing a growth curve. At various times samples were withdrawn for cell number per milliliter determination, ^{51}Cr uptake, and spontaneous cell lysis in 3 h. The mastocytoma grew exponentially with a doubling time of 10 h (Fig. 1). There was no difference between exponentially growing and stationary phase mastocytoma cells with

respect to ^{51}Cr uptake. However, the extent of spontaneous cell lysis became very pronounced in cells harvested from the stationary phase of growth. It was also found that stationary phase cells were significantly less susceptible to specific lymphocyte mediated lysis. (This phenomenon has recently been reported to exist in several mammalian tumors [14].) Hence, with the use of exponentially growing target cells, complete lysis, as indicated by 100% specific ^{51}Cr release, was consistently obtained within 4 h, the lymphocyte to target cell ratios being as low as 13/1 (Table I).

Second, we found that using RPMI 1640 medium in place of Dulbecco's modified Eagle's medium (9) and gently rocking the reaction mixtures greatly enhanced the efficiency of lymphocyte-mediated cytotoxicity (4).

Inhibition of Lymphocyte-Mediated Cytotoxicity by Isoantiserum.—Previous

TABLE I
*Effect of Lymphocyte/Target Cell Ratio on the Kinetics of ^{51}Cr Release**

Lymphocyte/target cell ratio	Specific ^{51}Cr release		
	90 min‡	180 min	270 min
		%	
1/1	2	15	23
7/1	19	60	78
13/1	32	78	100
22/1	52	86	100
43/1	53	94	100
100/1	87	90	100

* Incubations were done in RPMI 1640 medium containing 10% CS.

‡ Incubation period.

reports (1, 4) have indicated that heat-inactivated tumor-specific isoantiserum effectively inhibits the cytotoxicity of sensitized lymphocytes against their respective target cells. The results reported to date suggest that the observed inhibition is static.

We studied the effect of isoantiserum on the cytotoxic activity of sensitized lymphocytes. First, an experiment similar to that of Brunner et al. (1) was carried out to determine the dose of pooled antisera that gave maximal inhibition of LMC. Increasing amounts of heat-inactivated antiserum were added to incubation mixtures containing either sensitized or normal spleen cells. Samples were withdrawn at 90 min, and the ^{51}Cr released into the supernatant was determined. The control culture containing normal serum showed 86% specific lysis whereas that containing 10% sensitized mouse serum showed only 18% (Fig. 2, curve A). Antiserum concentrations of 3% and 6% showed 42% and 30% lysis, respectively. The inhibitory effect of the antiserum was found to be transient: samples removed at 180 and 270 min (Fig. 2, curves B and C)

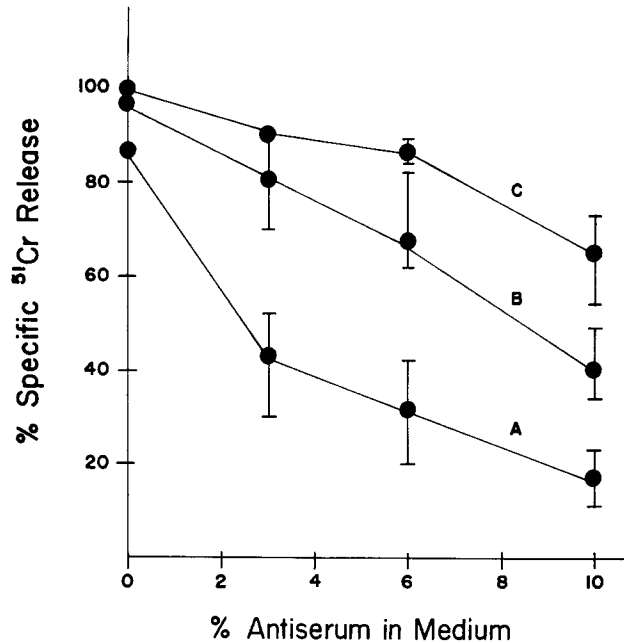


FIG. 2. Escape from isoantiserum inhibition of sensitized lymphocyte-mediated target cell lysis. Increasing amounts of antimastocytoma isoantiserum were added to 4 ml of reaction mixtures containing 1×10^7 sensitized or normal spleen cells and 1×10^5 ⁵¹Cr-labeled target cells/ml. Total serum concentration in the reaction mixture was brought to 10% with de-complemented CS. The reaction mixtures were distributed into three 1-ml aliquots for sampling at the times indicated: 90 min (curve A); 180 min (curve B); 270 min (curve C). Percent ⁵¹Cr release was monitored as described under Materials and Methods. Each point indicates the average of three to five experiments.

showed a significant time-dependent decrease in the inhibitory effect of the isoantiserum.

The blocking activity was found in a serum fraction precipitated with 33–55% $(\text{NH}_4)_2\text{SO}_4$ and separated with the 7S globulins on Sephadex G-200. These results suggested that we were working with a blocking antibody similar to that reported by Brunner and colleagues (1).

Several obvious explanations for the unblocking (escape) phenomenon had to be considered.

First, although the CS and IS were inactivated at 56°C for 45 min, the possibility of complement arising from the spleen or mastocytoma cell population and causing antibody-mediated lysis had to be investigated. Table II shows the results of the experiment testing this possibility. Neither de-complemented antiserum incubated with target cells nor IS combined with normal lymphocytes and target cells showed significant specific ⁵¹Cr release over

TABLE II
Inability of Depleted Isoantiserum to Effect Cytotoxicity

Contents of incubation mixture	Specific ⁵¹ Cr release*		
	90 min	180 min	270 min
		%	
Isoantiserum (IS) †	0	0	0
IS plus normal spleen cells	0	0	0
Normal mouse serum (NMS) plus sensitized spleen cells	51	84	90

* The data represent the average of three experiments.

† The serum concentration in the incubation mixtures was 10%, and the lymphocyte/target cell ratio was 100/1.

background during the course of our experiments. Moreover, 100 μ g/ml of carrageenan, a known complement inhibitor, did not effect the unblocking phenomena. These data argued against complement-mediated cytotoxicity. At the same time, the antibody-induced cytotoxic effect of normal lymphocytes (B cells) was found to be not significant in our assay system (15-17).

Secondly, in vitro activation of macrophages in response to antigen-antibody complexes had to be considered as a cause of lysis of target cells in the presence of antiserum (18, 19). This possibility was studied in the following manner. The sensitized spleen cells were incubated in plastic Petri dishes for 2 h at 37°C in a humidified incubator to separate adhering cells from nonadhering cells. The separated cells were tested for their cytotoxic effect. Adhering cells failed to exhibit a cytotoxic activity within 3 h. The nonadhering cells, however, exhibited the same cytotoxic activity as the original lymphocytes (Table III). The escape phenomenon was still evident.

Thirdly, it was felt that if the escape phenomenon was attributable to a minor lymphocyte population, reducing the lymphocyte to target cell ratio should dilute out such a population and abrogate the unblocking phenomenon. Table IV shows the results from experiments using three different lymphocyte to target cell ratios. It can be seen that a tenfold variation in lymphocyte to target cell ratio had no appreciable effect on the unblocking phenomenon. These results implied that the unblocking phenomenon was a function of the target cell rather than of the sensitized lymphocytes, suggesting that the target cells became refractory to isoantibody but sensitive to LMC as the incubation continued.

Escape from Antiserum Inhibition of LMC in the Presence of Excess Isoantiserum.—The escape from isoantiserum inhibition of the LMC may be a consequence of a nonspecific inactivation of the antiserum in our assay system. This possibility was investigated by adding fresh isoantiserum to the incubation mixture at the beginning of unblocking or by checking for residual blocking activity in the supernatant as follows: As shown in Table V, once unblocking

TABLE III
Escape from Isoantiserum Inhibition after Removal of Adhering Cells

	Specific ⁵¹ Cr release		
	60 min	120 min	180 min
Spleen cells		%	
CS	27	77	86
IS*	6	30	48
Nonadhering cells			
CS	2	89	100
IS*	10	28	54

* 6% isoantiserum brought to 10% serum with CS. In each case the lymphocyte/target cell ratio was 50/1. The data represent the average of two experiments.

TABLE IV
*The Effect of Lymphocyte/Target Cell Ratio on the Escape Phenomena**

Lymphocyte/target cell ratio	Specific ⁵¹ Cr release		
	90 min	180 min	270 min
		%	
13/1	4	17	50
43/1	3	28	68
100/1	11	37	73

* The data, which represent the average of three experiments, are 6% isoantiserum brought to 10% serum with CS.

TABLE V
*Effect of Additional Isoantiserum on LMC**

	Specific ⁵¹ Cr release		
	90 min	180 min	270 min
		%	
Normal mouse serum	10	42	62
Isoantiserum	15	48	70

* Target cell-lymphocyte suspensions were incubated with 6% isoantiserum for 75 min, at which time an additional 5% NMS or 5% IS were added to the incubation mixtures. The data represent the average of three experiments.

had been initiated, the addition of fresh antiserum did not cause blocking. Furthermore, blocking activity was still detected in the culture medium after 180 min of incubation in the presence of absence of mastocytoma (Table VI). These data support the conclusion that the target cell is somehow capable of becoming refractory toward antibody directed at its surface determinants while it remains sensitive to LMC.

We studied whether tumor cell metabolism was required for the escape from

TABLE VI
Residual Blocking Activity in Culture Supernatant*

Incubation period	Preincubation period						Control‡
	30 min		90 min		180 min		
	+M	-M	+M	-M	+M	-M	
			%				
90 min	20	20	22	26	23	24	57
180 min	45	65	59	55	53	54	85

* 8% antiserum incubated for 30 min, 90 min, and 180 min in the presence or absence of mastocytoma cells (+M, -M). At the times indicated, the cultures were spun down and the supernatants were checked for blocking activity by incubating with fresh mastocytoma cells and sensitized spleen cells. Samples were removed at 90 min and 180 min, and percent specific ^{51}Cr release was determined. The lymphocyte/target cell ratio was 100/1.

‡ The control reaction mixture contained 10% NMS.

the inhibition of antiserum. A method of manipulating biosynthesis in the target cell without affecting spleen cell cytotoxicity was sought to confirm this idea.

The Effect of Temperature on the Kinetics of Escape from Antiserum Inhibition of LMC.—Previous studies indicated that membrane biosynthesis could be affected by low temperature (20, 21). If the escape from IS inhibition were a function of target cell membrane metabolism, a significant temperature effect on the kinetics of escape from antiserum inhibition of LMC would be expected.

Chromium-labeled mastocytoma cells were preincubated at 0°C for 15 min before the addition of 6% IS. The cell suspension was then incubated an additional 30–50 min at 0°C in an ice-water bath. The antibody-coated cells were washed at 4°C with serum-free RPMI medium. The washed cells were then divided and placed in two aliquots for incubation, one at 37°C and the other at 0°C, for 40 min. At the end of the incubation, both cultures were equilibrated to 37°C and spleen cells were added to produce cytotoxic activity.

As shown in Fig. 3, the cells incubated at 0°C showed a reproducible 30–40 min lag in escape compared with the cultures incubated continually at 37°C. This result supports the hypothesis that active membrane metabolism of target cells is involved in the escape phenomenon.

DISCUSSION

We have found that sensitized lymphocytes are capable of overcoming isoantiserum (IS) inhibition of their cytotoxic activity (Fig. 2). We think that the escape from the blocking effect of IS is a consequence of antigenic modulation on actively metabolizing mastocytoma cells. This conclusion is derived from the experimental evidence outlined below: (a) The escape phenomenon is independent of complement-mediated cytotoxicity because cell lysis is negligible unless sensitized lymphocytes are added (Table II). (b) There is no evidence of activation of normal lymphocytes (B cells) mediated by antigen-

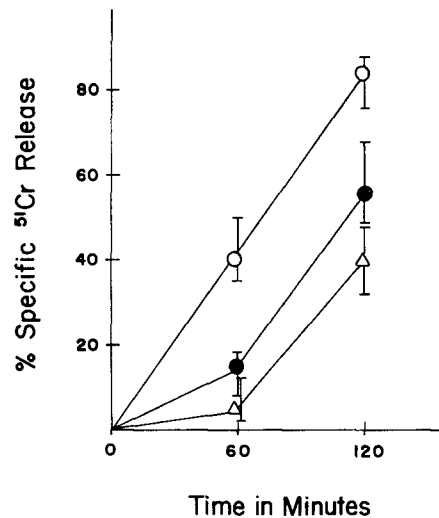


FIG. 3. Effect of low temperature incubation on the rate of escape of LMC from isoantiserum inhibition. ⁵¹Cr-labeled mastocytoma cells (sp act = 1.4×10^4 cpm/ 10^5 cells) suspended in serum-free RPMI 1640 were cooled in an ice-water bath for 15 min. Aliquots of the cooled cells were added to equal volumes of cooled medium containing 6% isoantiserum or normal mouse serum (NMS). The cells were allowed to incubate in the presence of the mouse serum for 30–50 min at 0°C. The antibody-coated cells were then collected and washed with serum-free RPMI by centrifugation at 350 *g* for 10 min at 0°C. The washed cells were divided into two groups: one was maintained at 0°C while the other was incubated at 37°C. At the end of a 40-min incubation at their respective temperatures, both suspensions were equilibrated to 37°C for 10 min, and the normal or sensitized spleen cells were added. The percent specific ⁵¹Cr release was determined as described under Materials and Methods. Normal mouse serum at 0°C and 37°C (○); 6% isoantiserum at 37°C (●); 6% isoantiserum at 0°C (Δ).

antibody complexes (Tables II and III) (15–17). Moreover, such nonspecific mechanisms of lymphocyte activation require 8–24 h, which is much longer than the incubation period used in our experiments. (c) Incubating antibody-coated target cells at 0°C produces a significant lag in the escape from IS inhibition (Fig. 3).

Brunner et al. (1) reported that the degree of inhibition by IS of LMC was related to the IS concentration in the incubation mixture. Our results in the early phase of incubation are in agreement with theirs on this point. However, we could demonstrate a time-dependent increase of LMC in the presence of excess antibody (Fig. 2).

Chang et al. (22) demonstrated that the amount of antibody adsorbed to strain A leukemia cells was proportional to the concentration of antisera in the reaction mixture. This study also revealed that 30% of the cell-bound antibody was released into the medium in an unaltered form within 2 h after removal of excess antibody.

The possibility of unmasking of antigenicity as a consequence of antibody release from the target cells must be considered as an explanation for our observation. It is assumed that antibody released, unaltered, from the cell surface (22) is in equilibrium with the cell-bound antibody if antibody is not limiting, as was the case in our experiments. If such an assumption is correct, our observation could be a result of competition between antibody and T cells on the target cells. The irreversible escape from IS inhibition (Tables V and VI) could be a consequence of a significantly higher affinity of T cells for the target cells than for the respective antibody.

Several investigators have reported evidence that fluidity of lymphocyte membranes may provide an escape from complement-mediated cytotoxicity. Maloney virus-transformed lymphoma cells lose the capacity to be destroyed by antibody during certain phases of the cell cycle (23). This complement refractory period was found to be directly proportional to the doubling time of the lymphoma cell line. Similarly, Takahashi et al. (24) and Lesley, Kettman, and Dutton (25) reported, respectively, that melanoma and thymus cells became refractory to complement-induced lysis if they were incubated at 37°C with antibodies before the addition of complement. Peripheral blood lymphocytes when incubated in medium containing antiserum against the IgG surface components proceeded to form a polar cap and to engulf the cell-bound antibody within 5–10 min when incubated at 37°C (21, 26). In view of these observations, we tried to explain our results as follows.

We think that actively metabolizing mastocytoma cells undergo a surface antigen modulation that leads to cells becoming refractory to the blocking effect of isoantibody but sensitive to the cytotoxic effect of sensitized lymphocytes. Unfortunately, direct demonstration of this conclusion requires an anti-metabolite that specifically inhibits mastocytoma cell membrane mobility. Support for this hypothesis has been sought by experiments showing that the escape phenomenon is dependent on temperature. The results obtained (Fig. 3) are reasonable when one considers that the "capping" phenomenon induced by antibody is also dependent on temperature (20, 21, 26). It appears that antigen recognition by specific T cells is not readily affected by events that significantly alter the efficiency of the humoral antibodies produced by B cells.

The Hellströms and their co-workers (27) have reported that tumor cells incubated with blocking serum regain sensitivity to the cytotoxic effects of immune lymphocytes within 6 h after incubation of the target cells in the absence of blocking serum. A possible reason for the disparity between their work and ours with respect to the rate of regained susceptibility to LMC may be related to differences in the doubling times of different cell lines.

In light of these findings, it is of current interest to determine whether or not the escape phenomenon we report is peculiar to an allogenic system. Our future studies will be directed toward examining the capacity of the humoral system to successfully protect foreign tissue from immune surveillance.

SUMMARY

Isoantiserum (IS) inhibition of lymphocyte-mediated cytotoxicity (LMC) was studied using an *in vitro* ^{51}Cr release assay system. In the early phase of incubation, LMC was competitively inhibited by IS. However, as the incubation continued, LMC irreversibly overcame IS inhibition (the "escape" phenomenon). Addition of fresh antiserum did not alter the course of the escape. Low-temperature incubation of isoantibody-coated target cells delayed the onset of the escape.

We have excluded the possibility that the escape phenomenon is induced by complement or by LMC mediated by antigen-antibody complex. It is hypothesized that antibody directed toward an actively metabolizing target cell induces an alteration in the cell membrane that alters further interaction with the antibody. However, sensitivity to lymphocyte cytotoxicity is maintained.

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