IN VITRO ACTIVATION OF CELLULAR IMMUNE RESPONSE TO GROSS VIRUS-INDUCED LYMPHOMA

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(Received for publication 1 May 1972)

Primary tumors in the autochthonous host and tumors transplanted into syngeneic recipients usually grow progressively despite the presence of detectable immunity to tumor-associated antigens. Several mechanisms have been suggested to explain this "escape" from immunological protection (1). Immunological enhancement by humoral antibodies is thought to be one of the major factors in the growth of antigenic tumors (1, 2). Most studies have emphasized the role of peripheral forms of inhibition. In afferent enhancement, antibodies bound to the tumor cells interfere with immunogenicity (3). In efferent enhancement, antibodies bound to antigenic sites protect the tumor from immunologic attack (2, 4, 5). There has been relatively little experimental evidence to support the central form of enhancement, in which immunological reactivity of sensitized cells is suppressed (6, 7). The relative in vivo roles of each of these mechanisms remains unclear.

Several in vitro studies of cell-mediated immunity have been performed to define the nature of the interference with immunological attack against tumor cells. As in the in vivo studies, most of the evidence pointed to a peripheral block (2, 8).

In previous studies (9, 10), we found that cellular immunity to a syngeneic Gross virus-induced lymphoma in W/Fu rats reached a peak at 9–10 days after tumor cell inoculation and then became undetectable after 20 days. The tumors themselves usually reached maximum size at 8–14 days and then completely regressed. The decrease in cellular reactivity occurred as the levels of 7S cytotoxic antibody rose (11). The question of antibody-mediated suppression of the cellular immune response was therefore raised. The present study was performed to determine whether there was some form of inhibition of the immune cell population, after it became unreactive in the direct cytotoxicity assays. Lymphocytes were placed under a variety of conditions in vitro, to determine if they could be reactivated to have cytotoxic effects against Gross tumor cells. Incubation of the directly unreactive immune cells for 12 hr or more at 37°C, in the absence of added antigen, was found to result in the ap-

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pearance of significant specific reactivity. Experiments were then undertaken to define the mechanism of this in vitro activation.

Materials and Methods

Animals.—Inbred, W/Fu strain, male rats were obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, Md.

Tumors.—The W/Fu rat lymphoma (C58NT)D (11), induced by Gross leukemia virus, was maintained in ascites form by serial intraperitoneal passage in weanling W/Fu rats. The sources and characteristics of the other tumors, used as specificity controls, have been described (12).

Immunization.—1×10^8 (C58NT)D tumor cells, suspended in saline, were injected subcutaneously in the right flank of 8–10-wk old W/Fu rats.

Harvesting of Lymphoid Cells.—(*a*) Spleens and lymph nodes were removed from normal or immunized rats and finely minced with scalpels in a few milliliters of balanced salt solution (BSS)[1] (Media Production Unit, National Institutes of Health, Bethesda, Md.) supplemented with 20% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N. Y.). The resultant single-cell suspensions were filtered by one passage through a cotton gauze sponge. (*b*) Peripheral blood was obtained by percutaneous cardiac puncture and mixed with heparin, 20 units/ml. Most of the erythrocytes were sedimented by adding 1 ml of plasmagel (Laboratoire Roger Bellon, Neuilly, France) per 3 ml of blood and incubating the mixture for 30 min at 37°C. The leukocyte-rich supernatant was then harvested. (*c*) Bone marrow was obtained from the femurs by rinsing of the marrow cavities with approximately 2 ml of BSS-FBS. The resultant single-cell suspensions were filtered through cotton gauze. (*d*) Peritoneal cells were obtained by introduction of 10–20 ml of BSS into the peritoneal cavity and then aspiration with a multiperforated 16-gauge trocar needle. Each of the cell suspensions was washed twice in 50 ml of BSS-FBS and then diluted to the final desired viable concentration in tissue culture medium, Eagle's minimal essential medium (Media Production Unit, National Institutes of Health) supplemented with 20% heat-inactivated FBS. Viability of all cell suspensions by trypan blue dye exclusion was 85–95%. In the direct cytotoxic assays, the cells were immediately tested for cytotoxic reactivity without any preincubation in vitro.

Purification of Lymphoid Cells.—Two methods were used to isolate the lymphocytes from the peripheral blood and from the lymphoid organs.

(*a*) *Nylon column*: 2 g of washed nylon (from a Fenwal Leukopak; Fenwal Labs., Morton Grove, Ill.) were packed into a 12 ml syringe. The column was first rinsed with warm (37°C) BSS-FBS. The cell suspension, at 1×10^7 cells/ml, was then passed through the column, at a flow rate of approximately 1–2 drops/sec.

(*b*) *Ficoll-Hypaque gradient*: Cell suspensions, at 1×10^7 cells/ml, were separated as described by Boyum (13). The top layer, containing the lymphocytes, was then washed twice in 50 ml of BSS-FBS.

Assay for Cell-Mediated Cytoxicity.—The assay was performed as previously described (9). The target cells, (C58NT)D, were harvested from the peritoneal cavity of weanling W/Fu rats. The cell suspension was adjusted to $1–2 \times 10^7$ cells/ml. 1 ml of this suspension was incubated with 150 μCi/ml ^{51}Cr (Radiochemical Center, Amersham, England) at 37°C and gently agitated for 30–40 min. For each experimental group, quadruplicate mixtures of lymphocytes (1×10^7) and labeled target cells (5×10^4), at an attacker to target cell ratio of 200:1, were incubated on a rocking platform for 4 hr at 37°C, with a moist atmosphere of 90% air, 10% carbon dioxide. The per cent cytotoxicity for each group was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{cpm } ^{51}\text{Cr released from cells during incubation}}{\text{cpm } ^{51}\text{Cr released from cells by three times freeze-thaw}} \times 100.$$

[1] *Abbreviations used in this paper*: BSS, balanced salt solution; FBS, fetal bovine serum.

Experimental results were expressed as:

% Cytotoxicity in experimental group − % cytotoxicity in control group.

Assay for Inhibition of Cytotoxicity.—Specificity of the cytotoxic reaction was tested by an inhibition assay, as previously described (14). To the usual incubation mixture of 1×10^7 lymphocytes and 5×10^4 ^{51}Cr-labeled (C58NT)D target cells, 0.2×10^6 unlabeled inhibitor cells were added to give a final attacker to target cell ratio of 40:1. The cytotoxicity assay was then performed in the usual manner.

In Vitro Activation of Lymphocytes.—Cell suspensions were prepared from the lymphoid cells of rats 40 days or longer after tumor cell inoculation. After washing twice with 50 ml of BSS-FBS, 1×10^7 cells in 1 ml of RPMI 1640 medium with 15% FBS in a 13 × 100 mm plastic tube were incubated for 18–24 hr at 37°C in an atmosphere of 10% carbon dioxide. After incubation, the cells were sedimented at 100 *g* for 10 min, washed once, and resuspended in RPMI 1640-FBS. The lymphocytes were then tested for cytotoxic reactivity against ^{51}Cr-labeled (C58NT)D cells, at the usual attacker to target cell ratio of 200:1. The concentration of attacker cells was calculated on the basis of viable cells, excluding trypan blue dye. Viability at the end of each of the incubation periods was 85–90%.

RESULTS

In Vitro Activation of Spleen Cells from Rats 40 Days after Tumor Inoculation.—Spleen cells obtained from rats 40 days after inoculation of (C58NT)D have never given positive results in the direct cytotoxicity assay. Two possible explanations for this unreactivity were studied: (*a*) The presence of 7S antibodies or other substances in the animals had an inhibitory effect on the effector cells. If this were the case, one might expect that spleen cells removed from the animal could become positive after a period of time. (*b*) At 40 days after tumor cell inoculation, cytotoxic cells were no longer present, perhaps because of lack of effective antigenic stimulation. Memory cells might then be present, which could be activated by exposure to antigen in the absence of circulating antibody (15, 16).

To test these possibilities, spleen cells were incubated in vitro for 24 hr at 37°C, by themselves or in the presence of (C58NT)D tumor cells (1×10^7 spleen cells + 5×10^4 tumor cells). The cells were then washed and tested against ^{51}Cr-labeled tumor cells. Table I gives the results of a typical experiment. In the direct assay, without preincubation, spleen-40[2] cells had no significant cytotoxic activity whereas spleen-10 cells were positive. After preincubation of spleen-40 cells for 24 hr, definite cytotoxic activity was found. Preincubation in the presence of antigen was not needed; in fact, the degree of cytotoxicity was slightly less in this group. As had been noted previously (10), spleen-10 cells incubated in the presence of antigen showed increased reactivity.

It was important to determine whether the cytotoxic reactivity which developed in the spleen-40 cells had the same immunological specificity as spleen-

[2] Immune lymphoid cells are designated by the source of the cells followed by the number of days after tumor cell inoculation; e.g., spleen-40 indicates spleen cells obtained 40 days after tumor inoculation.

10 cells. When activated spleen-40 cells were tested against LW-6, a spontaneous W/Fu rat leukemia, negative results were obtained (Table II). The specificity was further tested by an inhibition assay. Of the various cells tested, only (C58NT)D had inhibitory activity (Table II). This was the same specificity which had been previously demonstrated for spleen-10 cells (14).

TABLE I

Cytotoxicity of Spleen Cells after In Vitro Incubation for 24 hr

Cells in preincubation mixture	Period of preincubation	% Lysis (±SE) against ^{51}Cr-labeled (C58NT)D cells*
	hr	
Spleen-40	0	0.5 (0.4)
Spleen-40 + (58NT)D‡	0	−0.2 (0.5)
Spleen-40	24	9.8 (0.6)
Spleen-40 + (C58NT)D‡	24	8.6 (0.4)
Spleen-10	0	8.5 (0.3)
Spleen-10 + (C58NT)D‡	0	7.8 (0.4)
Spleen-10	24	8.1 (0.4)
Spleen-10 + (C58NT)D‡	24	16.5 (0.8)
Normal spleen	24	0.9 (0.5)
Normal spleen + (C58NT)D‡	24	1.3 (0.5)

* Control group = normal spleen cells without in vitro preincubation.

‡ At a ratio of 200:1, 1×10^7 spleen cells + 5×10^4 tumor cells.

TABLE II

Specificity of Cytotoxic Effects of Spleen-40, after Preincubation for 24 hr

Direct assay	Target cell	% Lysis (±SE)
	(C58NT)D	15.2 (0.6)
	LW-6	1.0 (0.7)

Inhibition assay — Inhibitor cells	% Lysis (±SE)*	Inhibitory effect on spleen-10 cells (14)
	20.2 (0.4)	
Rat tumors		
W/Fu (C58NT)D	7.1 (0.3)‡	+
W/Fu ERTh/V-G	8.9 (0.4)‡	+
W/Fu LW-6 (spontaneous leukemia)	19.4 (0.5)	−
Mouse cells		
C57BL/6 spleen	18.5 (0.6)	−
C57BL/6 E♂G2 (Gross virus-induced)	18.2 (0.7)	−
C57BL/6 EL-4 (chemically induced leukemia)	17.8 (1.0)	−
C57BL/6 RBL-5 (Rauscher virus-induced)	17.5 (1.1)	−
BALB/c LSTRA (Moloney virus-induced)	18.0 (0.8)	−

* Ratio of spleen-40 cells (1×10^7) to ^{51}Cr-labeled (C58NT)D cells (5×10^4) was 200:1. Inhibitor cells (0.2×10^6) were added to give a final attacker to target cell ratio of 40:1.

‡ Reduction by inhibitor cells significant, $P < 0.05$, by Student's t test.

In Vitro Activation of Other Lymphoid Cells.—It was previously found that cytotoxic reactivity of all of the lymphoid cells disappeared by 25 days after inoculation of (C58NT)D (10). It was of interest to determine whether cells from the various lymphoid organs could be activated in vitro, in the same manner as spleen-40 cells. Cells were harvested from the spleen, peripheral blood, lymph nodes, peritoneal cavity, and bone marrow of rats 40 days after inoculation with tumor cells. After 24 hr of preincubation at 37°C, the cells were washed and tested for cytotoxic reactivity against ^{51}Cr-labeled (C58NT)D cells (Table III). Spleen, peripheral blood, lymph node, and peritoneal cells all developed significant cytotoxic reactivity. The activity of spleen cells was always higher than that of the other lymphoid cells. Bone marrow cells did not become reactive.

Studies with Lymphocytes Obtained at Various Times after Immunization.—Spleen cells were obtained from W/Fu rats at various times after inoculation

TABLE III

Cytotoxicity of Various Lymphoid Cell Populations, after In Vitro Incubation for 24 hr

Attacker cells*	% Lysis (±SE)
Spleen	17.9 (0.6)‡
Blood	7.4 (0.8)‡
Lymph nodes	3.5 (0.7)‡
Peritoneal	4.7 (0.6)‡
Bone marrow	1.3 (0.8)

* Cells harvested from W/Fu rats, 40 days after immunization with (C58NT)D.

‡ Results significantly higher than controls, $P < 0.05$.

of (C58NT)D cells. Each of the cell suspensions was tested for direct cytotoxicity and also preincubated for 24 hr at 37°C and then tested for reactivity. These experiments were performed several times, with similar results on each occasion. None of the spleen cell preparations, from rats immunized 25 or more days earlier, were positive without preincubation. In vitro activation occurred, however, with most of the groups. The results of a typical experiment are summarized in Table IV. Spleen cells from rats 40 or more days after immunization became strongly positive after preincubation. In this experiment, as in all the others performed, higher results were seen in the groups which were 60 or more days after inoculation. In some instances, at 35–40 days after immunization, only low levels of activity developed. In the period between 25 and 35 days, no reactivity has been seen either on immediate testing or after preincubation.

Effect of Reinoculation of Tumor Cells upon Extent of In Vitro Activation.—It was previously shown that reexposure to tumor cells 30–40 days after initial immunization produced no detectable cytotoxic reactivity (9). It was of interest to determine whether a boost in cytotoxicity would become apparent after preincubation of spleen cells. At 27, 35, 60, and 100 days after initial

immunization, groups of three rats were given a second subcutaneous dose of 1×10^8 (C58NT)D cells. At 10 days after reinoculation, pooled spleen cells from each group were tested for immediate reactivity and for in vitro activation. For comparison, tests were also performed with pooled spleen cells from

TABLE IV

Cytotoxicity of Spleen Cells from Rats at Various Times after Immunization

Attacker cells; time after immunization	% Lysis (±SE)	
	Immediate test	After preincubation
days		
27	0.9 (0.5)	−0.6 (0.4)
33	0.5 (0.6)	−0.5 (0.4)
40	−0.4 (0.5)	13.3 (1.8)*
60	0.7 (0.8)	21.6 (1.3)*
80	−0.2 (0.4)	23.6 (1.5)*
120	0.8 (0.5)	27.8 (1.0)*
180	−0.3 (0.4)	16.7 (0.9)*

* Results significantly higher than controls, $P < 0.05$.

TABLE V

Effect of Reinoculation of Tumor Cells on In Vitro Activation

Attacker cells; time after initial immunization	Reinoculation*	% Lysis (±SE)	
		Immediate test	After 18 hr preincubation
days			
10	−	17.1 (0.8)	16.3 (1.7)
37	−	−0.8 (0.6)	16.2 (0.6)
37	+	−1.1 (0.8)	16.3 (0.5)
45	−	−2.4 (0.7)	12.9 (0.6)
45	+	−2.0 (0.8)	15.8 (1.0)‡
70	−	−0.1 (1.4)	20.6 (1.2)
70	+	0.5 (0.7)	24.7 (1.1)‡
110	−	−1.2 (0.9)	21.2 (0.7)
110	+	2.3 (0.8)	22.3 (0.7)

* 10 days before assay, some rats reinoculated subcutaneously with 1×10^8 (C58NT)D tumor cells.

‡ Significantly higher than group not receiving second injection of tumor cells, $P < 0.05$.

animals which were not reinoculated (Table V). On direct testing, cytotoxic activity was seen only in the spleen-10 group. After 18 hr preincubation, the spleen cells of all groups showed reactivity. In the 40 and 70 day groups, reinoculation resulted in significantly higher activity. In all of the groups, reinoculation of tumor cells resulted in no detectable tumor growth. This indicated that in vivo immune protection persisted for at least 100 days after initial tumor inoculation.

Studies to Define the Mechanism for In Vitro Activation.—Experiments were performed to determine the kinetics of activation of spleen-40 cells. Cells were incubated for various lengths of time at 37°C or at 4°C and then all tested at the same time for cytotoxic reactivity against (C58NT)D cells. Fig. 1 shows the results of preincubation at 37°C. A low but significant amount of activity appeared after 4 hr; peak reactivity was seen after 24 hr. A longer period of incubation resulted in a decline in activity. The cells incubated at 4°C remained viable but did not develop any detectable activity. This indi-

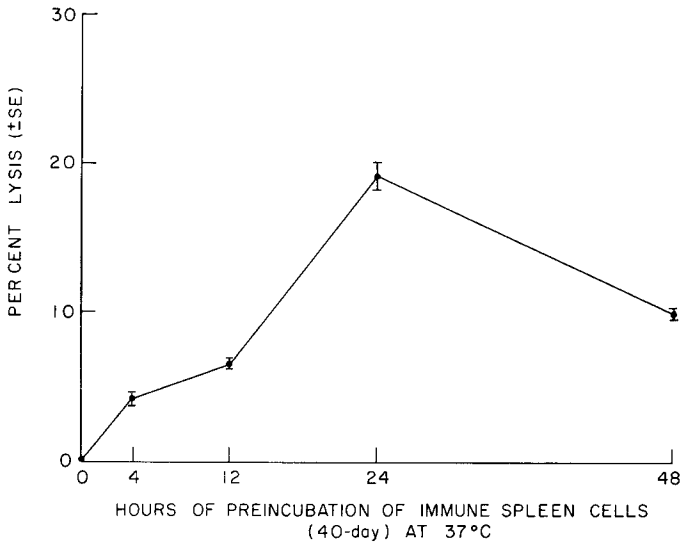

FIG. 1. Cytotoxic activity of spleen-40 cells against (C58NT)D target cells, after preincubation of immune cells at 37°C.

cated that in vitro activation is temperature dependent, and may depend on some active metabolic processes.

One possible explanation for the lack of immediate reactivity of spleen-40 cells was that an inhibitory substance was associated with the cells. Such an inhibitor might dissociate from the cells or become inactive after in vitro incubation at 37°C. The possible inhibitor was looked for in several different ways.

(*a*) Active spleen-10 cells were diluted with increasing amounts of spleen-40 cells and tested for cytotoxicity. The control was spleen-10 cells mixed with normal cells. Fig. 2 shows that both curves were very similar. The presence of spleen-40 cells had no inhibitory effects on the activity of the spleen-10 cells.

(*b*) If an inhibitor substance dissociated from the spleen-40 cells during in vitro incubation, it might be detectable in the supernatant fluid. After preincubation of spleen-40 cells for 24 hr, they were tested either in the preincu-

bation medium or in fresh medium, after washing the cells twice (experiment 1, Table VI). There was no significant difference between the results.

(*c*) An inhibitor was also looked for by suspending spleen-10 cells in the preincubation medium and testing for cytotoxic activity (experiment 2, Table VI). No inhibition was found.

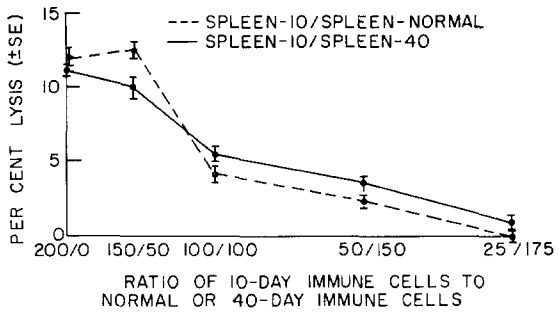

FIG. 2. Cytotoxic activity of spleen-10 cells mixed with varying proportions of normal spleen cells or spleen-40 cells. The total number of spleen cells added to the 5×10^4 (C58NT)D target cells was kept constant at 1×10^7.

TABLE VI

Tests for Possible Inhibitors in Preincubation Medium

Attacking cells	Medium used for assay	% Lysis (±SE)
Experiment 1		
Spleen-40, 24 hr preincubation	Fresh	12.8 (0.6)
" -40, 24 " "	Spleen-40 preincubation medium	12.5 (0.7)
Experiment 2		
Spleen-10	Fresh	11.1 (0.8)
" -10	Spleen-40 preincubation medium	12.9 (0.8)
Experiment 3		
Spleen-40, 24 hr preincubation in fresh medium	Fresh	16.5 (0.4)
Spleen-40, 24 hr preincubation in preincubation medium from an earlier experiment	Fresh	15.9 (0.7)

(*d*) Spleen-40 cells were incubated for 24 hr at 37°C in the preincubation medium of a previous experiment and then tested for cytotoxicity. This might be expected to subject the cells to a higher concentration of inhibitor. However, this did not result in a significant decrease in activity (experiment 3, Table VI).

(*e*) If an inhibitor substance were bound to the surface of spleen-40 cells,

it might be possible to remove it by trypsin digestion and thereby rapidly activate the cells. Spleen-40 cells were incubated for 30 min at 37°C with 0.25% crude trypsin or with 0.1% crystalline trypsin. The cells were then washed and tested for cytotoxic activity. Negative results were obtained.

Direct cellular reactivity declines and becomes undetectable at the time of

TABLE VII

*Effect of Rat Serum on In Vitro Activation**

Serum in preincubation medium	% Lysis (±SE)
Experiment 1	
25% FBS	16.4 (1.3)
25% normal rat serum (NRS)	4.9 (0.8)
25% immune serum, peak I‡	−1.4 (1.0)§
25% immune serum, peak II‖	−0.6 (1.1)§
Experiment 2	
25% FBS	14.4 (0.7)
25% NRS	4.5 (0.4)
25% immune serum, peak I	0.5 (0.3)§
25% immune serum, peak II	−0.5 (0.7)§
Experiment 3	
15% FBS	23.7 (1.5)
15% NRS	17.1 (0.7)
5% NRS, 10% FBS	21.1 (1.1)
1% NRS, 14% FBS	21.7 (1.0)
15% peak II	14.2 (1.2)
5% peak II, 10% FBS	16.8 (0.8)§
1% peak II, 14% FBS	17.0 (1.5)§

* Spleen-40 cells suspended in medium containing FBS or rat serum at various concentrations were preincubated at 37°C for 24 hr, washed once, and tested for cytotoxicity. As controls for each group, normal spleen cells were incubated in medium containing the same type and concentration of serum.

‡ Pooled serum from rats, 10–14 days after subcutaneous inoculation of 1×10^8 (C58NT)D cells. Cytotoxic antibody titer of serum was 60.

§ Activity significantly lower, $P < 0.05$, than that seen with normal rat serum at same concentration.

‖ Pooled serum from rats, 30–40 days after subcutaneous inoculation of 1×10^8 (C58NT)D cells. Cytotoxic antibody titer of serum was 160.

appearance of 7S antibodies (9). It seemed quite likely that the antibodies had an inhibitory influence on the immune lymphocytes. Incubation of the cells for a period of time in vitro, away from the influence of the antibodies, might allow them to become activated. The most direct way to test this hypothesis was to perform the in vitro incubation in the presence of rat sera containing 7S antibody (peak II) or 19S antibody (peak I serum). The results of three representative experiments are given in Table VII. None of the experiments gave clear-cut results. Complete inhibition of activation occurred

only when high concentrations of serum (25%) were used. At that level, normal rat serum also had inhibitory activity. There was, however, a significant difference between the effects of immune sera and of the normal sera. At lower concentrations of immune sera, only partial inhibition was seen and there were only small differences from the normal rat serum.

Effects of Separation of Lymphocytes on In Vitro Activation.—Experiments were performed to determine the nature of the cells involved in the in vitro

TABLE VIII

Effect of Lymphocyte Purification Techniques on In Vitro Activation

Attacker cells*	% Lysis (±SE)			
	No purification	Purification on nylon column		Purification on Ficoll-Hypaque gradient before preincubation
		Before preincubation	After preincubation	
Spleen-40	6.5 (0.4)	1.0 (0.5)	5.7 (0.8)	
Spleen-40	13.3 (1.8)	1.9 (1.2)	14.7 (1.1)	15.2 (0.9)
Spleen-40	11.8 (0.6)	0.7 (0.3)	13.0 (0.5)	
Spleen-80	23.6 (1.5)	0.7 (1.5)	22.5 (0.7)	25.4 (0.9)
Spleen-150	12.0 (1.4)	0.5 (1.0)	9.2 (0.9)	

* As controls for each group, normal spleen cells were manipulated in the same manner.

TABLE IX

Effect of X-Irradiation on In Vitro Activation

Treatment of attacker cells*	% Lysis (±SE)
No radiation	6.6 (0.3)
500 R, before preincubation	6.7 (0.4)
1000 R, " "	1.2 (0.4)
3000 R, " "	−0.6 (0.2)
500 R, after preincubation	7.1 (0.9)
1000 R, " "	8.1 (0.8)
3000 R, " "	7.3 (0.9)

* Spleen-40 cells preincubated at 37°C for 18 hr and then tested for cytotoxicity.

activation. Spleen-40 cells were passed through a nylon wool column and then incubated for 24 hr at 37°C. No cytotoxic activity was detectable in these cells (Table VIII). Several repeat experiments were performed, confirming these results. In contrast, lymphocytes isolated by centrifugation on a Ficoll-Hypaque gradient showed good in vitro activation. These experiments indicated that a cell type necessary for activation was removed by the nylon column. Experiments were then performed, to determine if, after in vitro activation, reactivity could still be removed by the nylon. No decrease in cytotoxicity was seen (Table VIII). As was previously found with spleen-10 cells (10), these experiments indicate that most active attacker cells do not adhere to nylon columns.

It seemed possible that normal phagocytic cells might be needed for activation to occur. Experiments were therefore performed in which equal numbers of column-separated spleen-40 cells and normal spleen cells were mixed. Incubation of these mixtures for 24 hr resulted in no appearance of cytotoxic activity.

Effect of X-Irradiation on In Vitro Activation.—To further define the characteristics of the cells involved in activation, the effects of X-irradiation were studied. When cells were exposed to 500 R before in vitro incubation, no inhibitory effect was seen (Table IX). Higher levels of radiation reduced or eliminated the appearance of cytotoxicity. When the cells were radiated with as much as 3000 R after activation, no inhibitory effects were observed.

DISCUSSION

Evidence has been presented here for some form of central inhibition of immune cell activity. Lymphoid cells from rats, 20 or more days after immunization, did not have any detectable direct cytotoxic activity. Incubation of these cells outside of the host's environment for 12–24 hr resulted in the appearance of considerable activity. The activated cells had the same immunological specificity as that observed with spleen-10 cells (14).

These findings are reminiscent of the study of McGregor et al. (17). They found that in vitro incubation of thoracic duct lymphocytes from rats tolerant to sheep erythrocytes resulted in their ability to produce hemolysin. However, they noted that a similar procedure failed to "break tolerance" to transplantation antigen.

Canty et al. (16) have observed a similar activation phenomenon with the cytotoxic reactivity to mouse alloantigens. Spleen cells from hyperimmune animals became much more reactive after transfer to sublethally irradiated syngeneic hosts. They postulated that the presence of humoral antibody in the intrasplenic environment of the primary hosts had a suppressive effect on immune cell activity. An important difference from the present study was that the increased activity only occurred when the immune spleen cells were administered to the secondary host along with antigen.

In the present study, we performed a variety of experiments to elucidate the mechanism for the in vitro activation. Removal of the immune cells from inhibitory substances was thought to be an important factor. Amos et al. (7) have postulated that a soluble inhibitor substance is the mediator of immunological enhancement. However, we found no evidence for an inhibitory activity in the spleen-40 cells or for release of an inhibitor into the supernatant fluid during the preincubation period. The possible role of humoral antibody was not clearly determined and the observed phenomena may therefore be different from classical immunological enhancement. The 7S cytotoxic antibody, which is present in vivo at the time of disappearance of direct cellular cytotoxic activity, did suppress the in vitro activation. However, peak I serum,

obtained from rats at the time of maximal direct cellular reactivity, had similar effects. Even normal rat serum had some suppressive activity.

The experimental observations which seemed to provide the most insight into the mechanism of activation were the effects of temperature and cell separation. The appearance of activity upon incubation in vitro occurred at 37°C but not at 4°C. This indicated that simple removal of the cells from the most environment was not sufficient, but rather that some active change had to occur in the lymphocytes. Sensitization or stimulation of lymphocytes by antigen carried over in the spleen cell suspension seems unlikely, however, because of the short time period needed for activation.

The activation process also had a requirement for cells which adhered to a nylon column. Once activation occurred, however, adherent cells were no longer needed. This could be accounted for in different ways.

(*a*) The immune cells might have been initially adherent, and during the period of incubation, the surface characteristics changed and they became less adherent. Active spleen-10 and spleen-40 cells did not appear to adhere significantly to the nylon. It is known that lymphoid cells can adhere to nylon or glass, but these cells have generally been characterized as bone marrow-derived lymphocytes (18). It is thought that thymus-derived lymphocytes are usually responsible for direct cytotoxic reactivity (19). However, the distinction between adherence of bone marrow- and thymus-derived lymphocytes does not appear to be absolute. It was recently reported that some thymus-derived cytotoxic peritoneal lymphocytes were adherent (20). In addition, the type of lymphocyte responsible for cytotoxicity in the present system or in other tumor-specific systems has not been clearly demonstrated.

(*b*) An adherent helper cell may be needed for in vitro activation. There are some studies which indicate that helper cells play a role in cell-mediated immunity as well as in humoral immunity (21–23). Grant et al. (23) have reported that adherent normal bone marrow cells, after exposure to immune thymus cells plus antigen, were able to inhibit the growth of an allogeneic tumor cell. A nonspecific helper activity of phagocytic cells is not a likely explanation, however, for the present observations, since the addition of normal spleen cells to the column-purified immune cells, before incubation for 24 hr, did not result in activation.

Another possible explanation for the activation phenomenon is that there may be an adherent cell in the immune lymphoid population which inhibits activation of effector cells. During the period of preincubation, the putative inhibitory cell may become inactive or die. Gowans (24) observed that, after in vitro incubation of thoracic duct lymphocytes for 24 hr at 37°C, most large lymphocytes died and over-all viability decreased to 78% or less. In our studies, however, viability of the lymphocytes after incubation was always 85% or greater, and there was no appreciable loss of large lymphocytes. The possible role of an inhibitor cell also does not account for the loss of activation

after nylon column separation. If an inhibitor cell was removed by the column, this should have resulted in increased activity rather than less. Experiments are now in progress to further characterize the cells involved in the activation process, in an attempt to distinguish among these possible mechanisms.

Any postulated mechanism for the in vitro activation would have to account for lack of activation of cells from rats 25–35 days after immunization. Also, the reasons for the differences in the activation of the various lymphoid cell populations need to be explained. These might be due to the number of potential effector cells in the different sites, or to the proportion of helper or inhibitor cells.

The occurrence of in vitro activation could explain many of the apparent differences among the various in vitro assays for cell-mediated cytotoxicity. The Hellströms (2), using a colony inhibition technique, have found cellular reactivity at various stages of tumor involvement and after tumor regression. In the colony inhibition assay, lymphocytes are incubated in vitro at 37°C for several days, an ample period for activation to occur. In comparative studies of reactivity against the rat Gross lymphoma with the present cytotoxicity assay and the microcytotoxicity assay as used by the Hellströms, we have indeed found that the differences in results could be accounted for by the activation phenomenon (Wright, Ortiz de Landazuri, and Herberman, unpublished observations). The results of Denham et al. (15) could also be explained in the same way. The characteristics of their 21-day spleen cells were quite similar to those of our spleen-40 cells.

SUMMARY

Spleen cells from W/Fu rats 40 days or more after immunization with a syngeneic Gross virus-induced leukemia were unreactive in direct cytotoxic assays. Incubation of these immune cells at 37°C for 12 hr or longer, in the absence of antigen, resulted in the appearance of specific cytotoxic reactivity. Other lymphoid cells from the immune rats also were activated upon in vitro incubation, but to a lesser extent. Experiments were performed to define the necessary conditions and the mechanism for the in vitro incubation. Activation was temperature dependent, occurring at 37°C but not at 4°C. Immune serum suppressed the activation, but normal rat serum also had some inhibitory activity. Passage of immune cells through a nylon column, before preincubation, prevented activation. In contrast, exposure to nylon after preincubation did not remove cytotoxic reactivity. These findings demonstrate the reversal of a central inhibition of immune cell activity. The explanations offered for this phenomenon included change in surface characteristics of the immune cells during in vitro incubation, and the possible need for an adherent helper cell.

The authors wish to thank Myrthel Nunn, Hilda Porter, and John Hoffman for their excellent technical assistance.

BIBLIOGRAPHY

1. Klein, G. 1969. Experimental studies in tumor immunology. *Fed. Proc.* **28:**1739.
2. Hellström, K. E., and I. Hellström. 1970. Immunological enhancement as studied by cell culture techniques. *Annu. Rev. Microbiol.* **24:**373.
3. Snell, E. D., H. J. Winn, J. H. Stimpfling, and S. J. Parker. 1960. Depression by antibody of the immune response to homografts and its role in immunological enhancement. *J. Exp. Med.* **112:**293.
4. Möller, G. 1963. Studies on the mechanism of immunological enhancement of tumor homografts. II. Effect of isoantibodies on various tumor cells. *J. Natl. Cancer Inst.* **30:**1177.
5. Berne, B. H. 1965. Immunological homograft enhancement. Interactions of antiserum and skin and tumor homografts. *Proc. Soc. Exp. Biol. Med.* **118:**228.
6. Takasugi, M., and W. H. Hildemann. 1969. Regulation of immunity toward allogeneic tumors in mice. II. Effect of antiserum and antiserum fractions on cellular and humoral response. *J. Natl. Cancer Inst.* **43:**857.
7. Amos, D. B., I. Cohen, and W. J. Klein, Jr. 1970. Mechanism of immunologic enhancement. *Transplant. Proc.* **2:**68.
8. Mauel, J., H. Rudolf, B. Chapuis, and K. T. Brunner. 1970. Studies of allograft immunity in mice. II. Mechanism of target cell inactivation in vitro by sensitized lymphocytes. *Immunology.* **18:**517.
9. Oren, M. E., R. B. Herberman, and T. G. Canty. 1971. Immune response to Gross virus-induced lymphoma. II. Kinetics of the cellular immune response. *J. Natl. Cancer Inst.* **46:**621.
10. Ortiz de Landazuri, M., and R. B. Herberman. 1972. Immune response to Gross virus-induced lymphoma. III. Characteristics of the cellular immune response. *J. Natl. Cancer Inst.* **49:**147.
11. Herberman, R. B., and M. E. Oren. 1971. Immune response to Gross virus-induced lymphoma. I. Kinetics of cytotoxic antibody response. *J. Natl. Cancer Inst.* **46:**391.
12. Herberman, R. B. 1972. Serological analysis of cell surface antigens of tumors induced by murine leukemia virus. *J. Natl. Cancer Inst.* **48:**265.
13. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** (Suppl.)**:**77.
14. Ortiz de Landazuri, M., and R. B. Herberman. 1972. Specificity of cellular immune reactivity to virus-induced tumors. *Nature New Biol.* **238:**18.
15. Denham, S., C. K. Grant, J. G. Hall, and P. Alexander. 1970. The occurrence of two types of cytotoxic lymphoid cells in mice immunised with allogeneic tumour cells. *Transplantation.* **9:**366.
16. Canty, T. G., J. R. Wunderlich, and F. Fletcher. 1971. Qualitative and quantitative studies of cytotoxic immune cells. *J. Immunol.* **106:**200.
17. McGregor, D. D., P. J. McCullogh, and J. L. Gowans. 1967. The role of lymphocytes in antibody formation. I. Restoration of the hemolysin response in x-irradiated rats with lymphocytes from normal and immunologically tolerant donors. *Proc. R. Soc. Lond. B Biol. Sci.* **168:**229.
18. Plotz, P., and N. Talal. 1967. Fractionation of splenic antibody-forming cells on glass bead columns. *J. Immunol.* **99:**1236.
19. Cerottini, J.-C., A. A. Nordin, and K. T. Brunner. 1971. Cellular and humoral

response to transplantation antigens. I. Development of alloantibody-forming cells and cytotoxic lymphocytes in the graft-*versus*-host reaction. *J. Exp. Med.* **134**:553.

20. Brunner, K. T., and J.-C. Cerottini. 1971. Cytotoxic lymphocyte as effector cells of cell-mediated immunity. *In* Progress in Immunology. D. B. Amos, editor. Academic Press, Inc., New York. 385.
21. Lonai, P., and M. Feldman. 1971. Cooperation of lymphoid cells in an in vitro graft reaction system. The role of the thymus cell. *Transplantation.* **10**:372.
22. Asofsky, R., H. Cantor, and R. E. Tigelaar. 1971. Cell interactions in the graft-*versus*-host response. *In* Progress in Immunology. D. B. Amos, editor. Academic Press, Inc., New York. 369.
23. Grant, C. K., G. A. Currie, and P. Alexander. 1972. Thymocytes from mice immunized against an allograft render bone-marrow cells specifically cytotoxic. *J. Exp. Med.* **135**:150.
24. Gowans, J. L. 1962. The fate of parental strain small lymphocytes in F_1 hybrid rats. *Ann. N. Y. Acad. Sci.* **99**:432.