LYMPHOCYTE-MEDIATED CYTOTOXICITY IN VITRO

Effect of Enhancing Antisera*

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The ability to construct in vitro models of biologic events has lead to considerable knowledge about the immune response. Govaerts (1) and Rosenau and Moon (2) were among the first to describe feasible in vitro systems for the study of cell-mediated immunity (CMI)[1] and their reports initiated much of the subsequent activity in this area. Since then, models for the study of CMI or of delayed hypersensitivity (DH) have been the subject of considerable effort and a number of promising in vitro models have been proposed. The subject of these models can be divided broadly into the following groups: (*a*) elaboration of mediator factors by cells involved in delayed hypersensitivity including transfer factor (3), migration-inhibition factor (4), proliferation-inhibition factor (5), transforming or blastogenic factor (6), lymphotoxin (7), chemotactic factors (8), and interferon (9); (*b*) cellular reactions (clonal proliferation, blast transformation, stimulation of deoxyribonucleic acid [DNA] synthesis) to antigens or mitogens (phytohemagglutinin [PHA], poke weed mitogen) in lymphocyte culture (10–12); and (*c*) target cell destruction by the activated lymphocyte (13–15). This last experimental system dealing with target cell destruction by lymphocytes has provided an impetus to the study of mechanisms of graft rejection and their modification.

One manner of modifying the rejection of a graft in vivo has been through the use of immune serum with specificities directed against the graft (16). Application of this phenomenon, termed enhancement, has effected prolonged or indefinite survival of grafts which otherwise would have been rejected (17–19). Until recently the study of enhancement has been restricted largely to in vivo models although notable exceptions do exist (see Discussion).

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[1] *Abbreviations used in this paper:* CMI, cell-mediated immunity; DH, delayed hypersensitivity; DNA, deoxyribonucleic acid; ILNC, immune lymph node cells; LCT, lymphocyte-mediated cytotoxicity; LNC, lymph node cells; NLNC, normal lymph node cells; NMS, normal mouse serum; PHA, phytohemagglutinin.

Lymphocyte-mediated cytotoxicity (LCT) in vitro has been used as a model for exploring the effects of normal serum, immune serum, or serum from tumor-bearing animals on CMI. To date, however, it has not been demonstrated specifically that enhancement in vivo and inhibition of LCT in vitro are the same phenomenon dependent upon the same mechanisms and factors. It is reasonable to assume that these are similar expressions of the same process and as such would depend upon identical mechanisms and serum factors. It is the purpose of the experiments reported here to test this hypothesis.

In a previous study in this laboratory (20) it was shown that enhancement was mediated by antigen-antibody complexes and that these complexes exerted an effect on the lymph node cells (20, 21). These cells could be obtained either from immunized or from unimmunized animals. At this point, it seemed appropriate to extend these observations to an in vitro model in which one could simplify the number of reactants and determine whether or not the same sera which were active in vivo were also active in vitro. Further, the role of antigen-antibody complexes could be tested in vitro in the light of the previous work demonstrating the participation of these complexes in the mediation of enhancement in vivo. We describe here an in vitro model for studying lymphocyte-mediated cytotoxic effects on tumor cells and report the ability of microliter volumes of antisera with known enhancing activity in vivo to be active in this in vitro model. The action both in vitro and in vivo is dependent upon the formation of antigen-antibody complexes.

Materials and Methods

Mice, Tumor, Sera, and Immunization.—The anti-BP8 sera, the BP8 ascites tumor, and the mouse strains employed have all been characterized and described previously (20). All sera were heated (56°C for 30 min) before use. Briefly, BP8 is an ascites tumor which is histocompatible for C3H (H-2k) mice and the tumor is maintained by weekly intraperitoneal inoculation into these mice. C57BL mice are resistant to the tumor, being H-2b. These latter mice were used as donors of normal or immune lymph node cells (ILNC). Immunization was performed by injecting 10^6 cells subcutaneously in each limb and in each lateral chest wall (total, 6×10^6 cells), or mice were immunized intravenously by injecting $2–3 \times 10^6$ cells into the lateral tail vein. Control mice were injected subcutaneously with 0.02 ml of complete Freund's adjuvant into each limb and each lateral chest wall.

Tissue Culture Media.—The basic medium was RPMI-1640, each 100 ml of which was supplemented with 20 mg of L-arginine, 0.2 mM of L-glutamine, 10,000 units of potassium Penicillin G, 10 mg of ampicillin, and 10 ml of IPT-fetal calf serum (heated to 56°C for 30 min). RPMI-1640, glutamine, and IPT-fetal calf serum (certified free of gamma globulin, tested by immunoprecipitation) were obtained from Grand Island Biological Company, Grand Island, N. Y. The arginine solution was prepared from the dry reagent (Schwarz Bio Research Inc., Orangeburg, N. Y.). Penicillin G (Pfizer Lab. Division, New York) and ampicillin (Ayerst Laboratories, New York) were standard pharmaceutical preparations for human use.

Culture Conditions.—Cells were cultured in screw-capped 16- × 100-mm sterile tubes. These were incubated vertically in a 30°C water bath without shaking or rocking. The standard culture volume was 2 ml, although in a few instances, 1-ml cultures were used. Tubes

were autoclaved immediately before use. A series of experiments was carried out at temperatures of 26°, 28°, 30°, 32°, 34°, and 37°C to determine the temperature optimum for this culture system. It was found that 30°C yielded the most reproducible, reliable system. 10^6 tumor cells were used/ml of culture and concentrations of lymph node cells (LNC) ranged from 1 to 24 $\times$ 10^6/ml. Concentrations of spleen cells ranged from 5 to 80 $\times$ 10^6/ml.

Preparation of Cell Suspensions.—For all preparation and dissection, sterile glassware, instruments, and solutions were used throughout.

Tumor: A tumor-bearing C3H mouse was killed by cervical luxation and mounted abdomen up on an operating board. The fur was thoroughly wetted with 70% alcohol and with the aid of fine scissors the skin was reflected back to expose the peritoneal surface. Then a clamp was fastened to the linea alba and the peritoneum was tented upward with traction on the clamp. A syringe and needle were used to draw the tumor cell-containing ascites fluid from the abdomen. This was placed in a capped test tube and washed three times with barbital buffer (pH 7.4). After determining the tumor cell concentration, a suspension of 4 or 8 $\times$ 10^6 tumor cells/ml was prepared in culture medium. Aliquots of 0.5 or 0.25 ml were dispensed to each culture tube. This yielded a total of 2 $\times$ 10^6 tumor cells.

Lymphoid cells: Subcutaneously immunized or normal mice were killed and mounted for dissection as above. The skin was reflected and the axillary, brachial, and inguinal lymph nodes were removed and placed in a Petri dish. Mice that had been immunized intravenously were treated similarly but, in addition, the mesenteric and cervical lymph nodes were removed and added to the other nodes. Spleens were removed and placed in a separate Petri dish. Single cell suspensions were prepared by teasing the lymph nodes apart with a 20 gauge needle and fine forceps. Spleen cells were prepared by incising the capsule and gently expressing the contents by kneeding the spleen with the flat part of a small scissors. Both the LNC and spleen cells were then expressed gently through a 22 then a 25 gauge needle. After this, the cells were washed three times in medium. Cells were counted in a Neubauer chamber with trypan blue exclusion used to determine cell viability. At least 90% of cells were usually viable and cell suspensions were not used if less than 90% of the cells were viable.

Cell Enumeration.—At various time intervals, samples were withdrawn from the culture and the per cent of viable cells determined. The time periods at which samples were taken varied from 0 to 40 hr. The tubes were mixed well and 0.1 ml of the culture suspension was withdrawn using an Oxford pipette and sterile plastic tip. This was added to 0.1 cc of 0.3% trypan blue and the total number of cells and the number of dead cells were determined in a Neubauer chamber. Results are expressed either as the per cent of viable tumor cells or the per cent of dead tumor cells. Per cent of dead tumor cells is also referred to as per cent of cytotoxicity. The results are given for the cells in culture at a given sample time. Differentiation of tumor cells from LNC is quite easy as the tumor cells are at least three to four times as large as the LNC (22).

Interaction of Antibody and Tumor Cells.—In the first set of experiments, 1 or 10 μl of an anti-BP8 antiserum (the designations for the two different anti-BP8 antisera with known enhancing activity in vivo are "BH" and "669") were added to test cultures containing ILNC and tumor cells. Similar quantities of normal mouse serum (NMS) which had been heated (56°C for 30 min) were added to control cultures with the same number of ILNC and tumor cells. In the second set of experiments, 10 μl of an anti-BP8 antiserum were added to 0.2 ml of tumor cell suspension (2 $\times$ 10^6 tumor cells) and 0.8 ml of medium (in duplicate). The mixture was allowed to incubate at room temperature for 45 min. The cells were sedimented by centrifugation and the supernatant saved. The cells were washed twice with cold (4°C) medium and retained. These are referred to as washed tumor-antibody complexes. To the supernatant was added 0.25 ml which contained 46 $\times$ 10^6 tumor cells, and the mixture was allowed to incubate for 45 min at room temperature. The cells were then sedimented and the supernatant saved as "absorbed antibody." This entire volume was added to a culture of immune lymph node cells and tumor cells.

10 μl of antibody were allowed to incubate with immune lymph node cells for 45 min as in the first step above. The lymph node cells were then washed twice and used in the LCT assay as a control for the immune lymph node cells that had been exposed to tumor-antibody complexes (above).

Test for Lymphotoxin Activity.—The methods of Granger et al. (7, 23, 24) were employed for collecting and storing supernatants from cultures of tumor cells with immune or normal LNC, tumor cells with immune spleen cells, or from cultures of tumor cells alone. They were tested on HeLa cell monolayers.

RESULTS

Normal and Immune C57BL LNC.—Fig. 1 shows the results of culturing BP8 tumor cells alone or in combination with normal or immune C57BL LNC. In

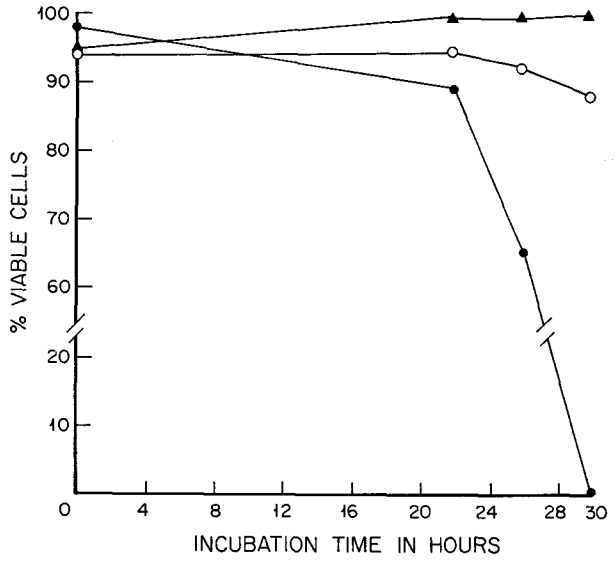

FIG. 1. Per cent viable tumor cells remaining in cultures of (▲—▲) tumor cells only ○—○, tumor cells with normal C57BL LNC; ●—●, tumor cells with immune C57BL LNC

this experiment, immune LNC came from animals killed 5 days after subcutaneous immunization and were cultured at a concentration of 13.5 × 10^6/ml. In other experiments, immune LNC were employed at concentrations ranging from 1 × 10^6/ml to 24 × 10^6/ml (see below). Normal LNC (NLNC) were employed at concentrations up to 40 × 10^6/ml. All concentrations of normal LNC gave similar results and the curve shown is for the highest concentration. The maximum cytotoxicity observed with normal LNC was 21% (37 × 10^6 NLNC/ml). With the exception of this single experiment, seven other assays with NLNC gave 10% cytotoxicity or less. The mean cytotoxicity observed for all experiments with NLNC was 6.4 with a standard error of 2.35%.

Control Cell Suspensions.—Fig. 2 depicts the results obtained when normal

C3H LNC, C3H LNC from tumor-bearing animals, and LNC taken from animals 5 or 7 days after injection with Freund's adjuvant were tested against tumor cells in this system (the results are shown for LNC taken from animals 5 days after injection of Freund's adjuvant, but the results from animals 7 days after immunization are not different). It can be seen that none of these control LNC is capable of effecting tumor cell cytotoxicity in this system. The mean cytotoxicity observed in all experiments using these control cell suspensions was 5.2% with a standard error of 1.6%. Immune (5 or 7 day postinjection) or nor-

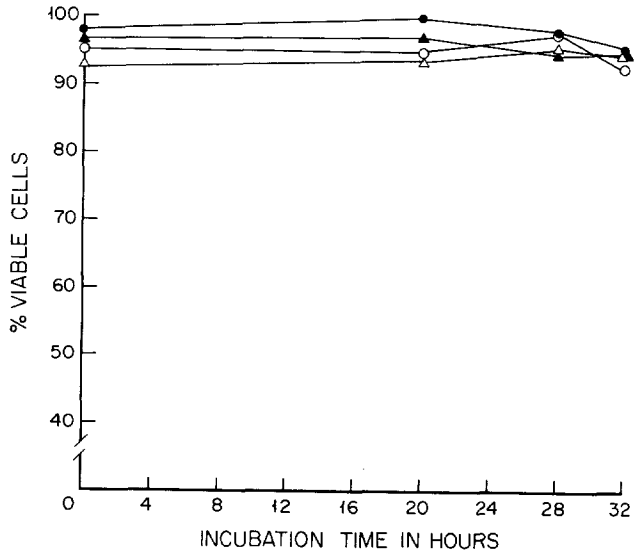

FIG. 2. Per cent viable tumor cells remaining in cultures of (▲—▲) tumor cells only; ●—●, tumor cells with normal C3H LNC; △—△, tumor cells with LNC from tumor-bearing C3H; ○—○, tumor cells with LNC from C57BL 5 days after immunization with Freund's adjuvant.

mal C57BL spleen cells are also incapable of expressing cytotoxicity in this system.

Kinetics of Cytotoxicity.—The results of studying the kinetics of this reaction are shown in Fig. 3. Samples were taken at 0, 2, 4, 8, 12, 14, 16, 20, 24, 28, and 30 hr after the initiation of the culture. In this experiment, as in others, definite cytotoxicity did not appear until after the 19th hr of culture and progressed rapidly thereafter. Maximum cytotoxicity was reached usually by the 26th–30th hr. After initial experiments in which the time course of the cytotoxic reaction was studied and defined, subsequent experiments employed two to three samples taken after 18 hr of culture in order to determine the presence or absence of LCT. Incubation of control lymphocytes with tumor cells for longer periods of

time (40 hr) did not alter the results. The addition of guinea pig complement to normal or immune LNC cultures did not change the per cent of dead cells and did not alter the kinetics.

Lymphocyte:Target Cell Ratio.—Fig. 4 illustrates the results obtained with various lymphocyte:target cell ratios. The ILNC were obtained from animals 5 days after immunization. Since the concentration of tumor cells is 1×10^6/ml, the ratio is also the concentration (in millions) of LNC per milliliter. Cytotoxicity was observed at ratios as low as 4.5 to 1, but ratios of 10:1 up to 24:1 were used in order to obtain reproducible results. Nine separate assays (each assay tested one or more of six concentrations of ILNC) in duplicate have been per-

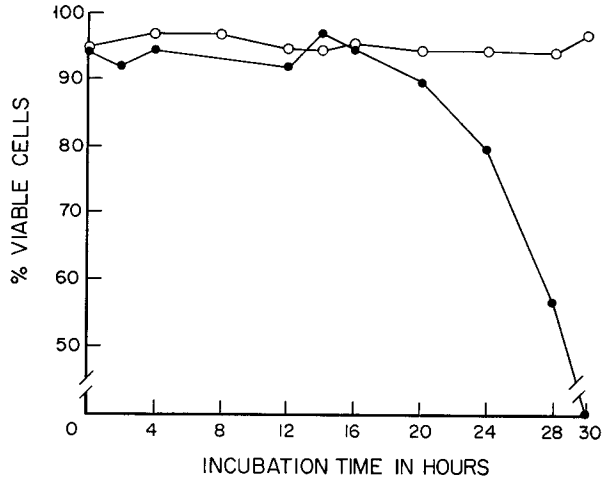

FIG. 3. Kinetics of cytotoxicity. Per cent viable tumor cells remaining in cultures of (○—○) tumor cells only; ●—●, tumor cells with 12×10^6 ILNC/ml.

formed in this range (10-24:1) with similar results (See Table I). All experiments were carried out with LNC from animals killed 5 or 7 days after subcutaneous immunization.

The Activity of LNC Obtained at Various Times after Immunization.—Animals were killed at 3, 5, 7, 9, 10, 11, 14, and 15 days after immunization and the LNC removed and tested for cytotoxic activity against the tumor cells. The results are shown in Fig. 5. Each experiment was performed at least twice. LNC are most active at 5 or 7 days postimmunization and yield 100% cytotoxicity consistently. There is no detectable cytotoxicity at days 3, 9, 11, 12, or 14. However, at 15 days after immunization there appears to be some measurable increase in LCT activity (average of two experiments) as seen in Fig. 5.

Effect of Enhancing Sera.—The next experiments were designed to test the effect of known enhancing antisera on LCT in vitro. Both sera (see Materials

and Methods section on interaction of antibody and tumor cells) used in these experiments have been shown previously to produce enhancement in vivo (20, 21). Microliter quantities of these antisera were capable of abrogating lymphocytotoxic activity in vitro. The results are shown in Fig. 6 for antisera BH and were not different for the other antiserum, No. 669. The addition of antisera No. 669 to cultures of ILNC and tumor cells resulted in 87% viable cells at 29 hr of culture, whereas in the mixture of ILNC and tumor cells alone there were 19% viable cells at this same time. Absorption of the sera with fresh BP8 tumor cells

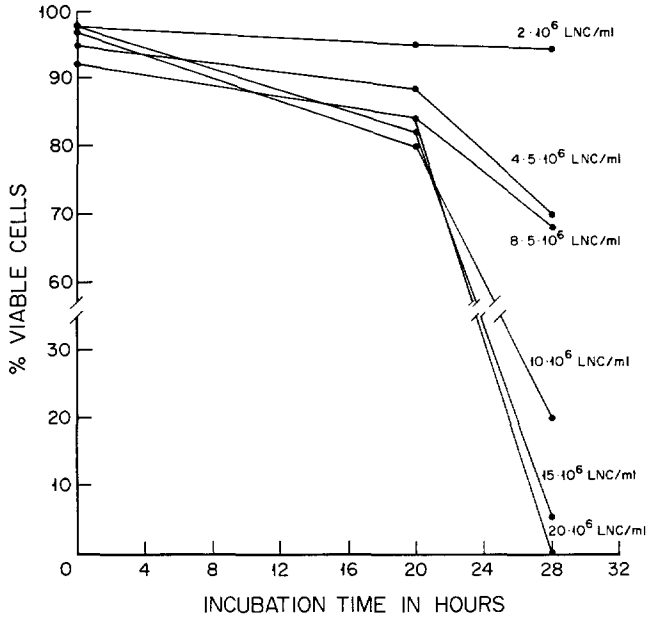

FIG. 4. Per cent viable tumor cells remaining in cultures of tumor cells with various concentrations of immune LNC.

before testing eliminated the ability to suppress in vitro cytotoxicity. The inhibition of LCT occurred when the antisera were added to the final culture mixture of ILNC and tumor cells. More notably, inhibition of lymphocytotoxic activity was seen when washed tumor-antibody complexes were mixed with the ILNC and cultured. In these experiments the tumor cells are treated with antibody, washed, and then added to the ILNC. In order to determine whether the antibody-antigen complexes were affecting the lymphoid cells, the following control procedure was performed. Antibody was incubated with ILNC first, the lymphoid cells were then washed, and these cells were added to the tumor cells. No inhibition of ILNC cytotoxic activity was observed in these experiments.

Most significantly, however, lymphocytes which were incubated with tumor-

antibody complexes, washed, and then added to fresh tumor cells which had not been treated with antibody were also incapable of exhibiting LCT. Normal mouse serum had no effect on LCT.

Intravenous Immunization.—In another series of experiments an alternate method of immunization, namely the intravenous route, was tested. Cell suspensions were obtained from the lymph nodes of mice killed 3, 5, 7, 9, 10, or 12 days after intravenous immunization. A maximum LCT of 30% was obtained with lymphoid cell:tumor cell ratios of 20:1, using lymph nodes from mice killed 12 days after immunization. No cytotoxicity of tumor cells was observed using lymph node cells obtained from 3 to 9 days after intravenous immuniza-

TABLE I

Result of Various Lymphocyte:Tumor Cell Ratios on Tumor Cell Viability

No. of ILNC/ml* (x 10^{-6})	Average per cent viable tumor cells
24 (1)	0
20 (3)	4
18 (2)	13
15 (2)	10
14 (4)	2
10 (4)	5
8 (2)	48
5 (2)	65
2 (3)	96
0 (9)	98

* All ILNC were obtained 5 or 7 days after immunization. All cultures had 10^6 tumor cells/ml. Number of experiments shown in parentheses.

tion. The use of LNC obtained 10 days postimmunization at concentrations of 12 and 7.2 $\times$ 10^6/ml resulted in 22% cytotoxicity and less than 5% cytotoxicity, respectively.

Comparison of Events In Vivo and In Vitro.—In order to evaluate the relationship of the events in vitro to those in vivo, a different and very virulent strain of BP8 tumor which had arisen from the original strain was employed. Whereas the C57BL mice previously were tolerating 10^6 cells intraperitoneally, this particular BP8 tumor was lethal at this dose. Even at doses of 2 $\times$ 10^5 cells, approximately 25% mortality was being observed. This strain of tumor was used for immunizing mice and for testing in the LCT assay described here. In eight different experiments performed in duplicate, no cytotoxic activity was present in lymph node cells obtained from mice 5–7 days after subcutaneous immuniza-

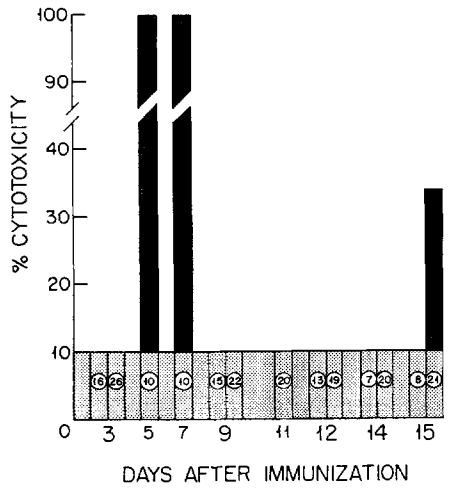

FIG. 5. Per cent dead cells (% cytotoxicity) observed after 30 hr of culture of tumor cells and ILNC. The ILNC were obtained on the indicated days after immunization. The circled numbers in the bars indicate the concentration of ILNC per milliliter of culture medium. The lightly stippled area indicates the control range. Where the bar does not extend beyond the control range, there was 10% cytotoxicity or less.

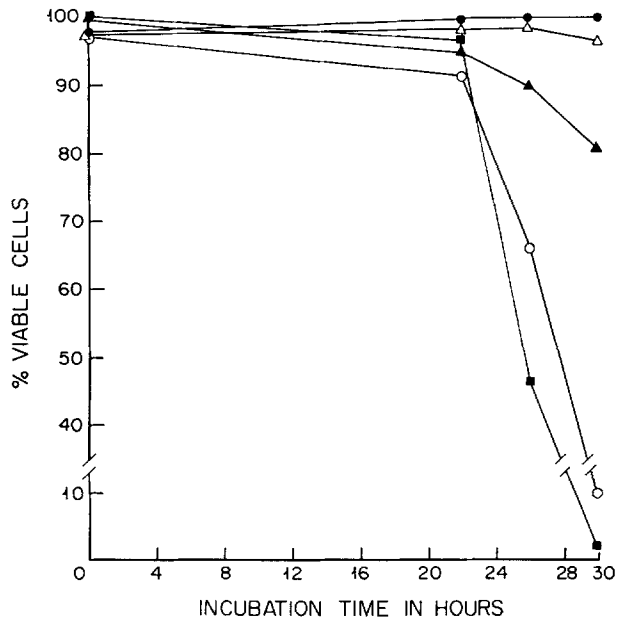

FIG. 6. Effect of antisera with known enhancing activity in vivo on LCT. Per cent viable tumor cells remaining in cultures of (●—●) tumor cells only; ○—○, tumor cells with ILNC; ▲—▲, tumor cells with ILNC plus 10 μl of "BH" antisera; △—△, tumor cells with ILNC plus 1 μl of "BH" antisera; ■—■, tumor cells with ILNC plus 10 μl of NMS.

tion. The results of these experiments are shown in Fig. 7. Thus, whatever the change in the tumor may have been, increased virulence in vivo was paralleled by a loss of the ability of the lymph node cells from immunized animals to express LCT in vitro.

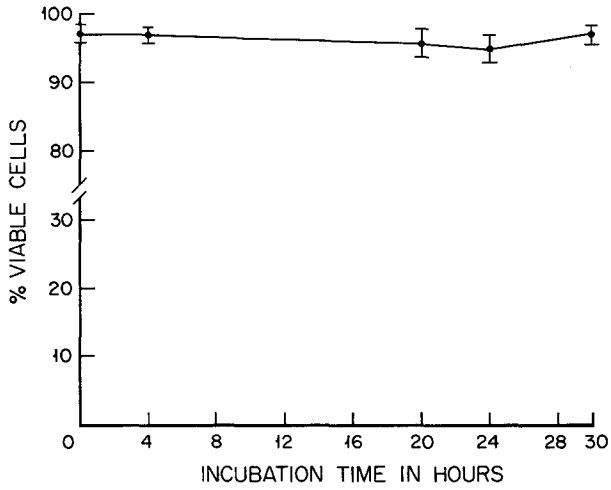

FIG. 7. Per cent viable tumor cells remaining in cultures of "virulent" tumor cells with ILNC. Results are shown as the mean ± the standard error for eight experiments in duplicate.

TABLE II

Counts Per Minute of Amino Acid-^{14}C Incorporated into Protein

Source of supernatant	Mean ± range/2	Amount of protein ^{14}C synthesized (expressed as per cent of control)
		%
Tumor cells alone	760 ± 39	100 (control)
Tumor cells + ISPC* (40×10^6/ml)	664 ± 82	87
" " + NLNC (20×10^6/ml)	812 ± 187	107
" " + ILNC (19.4×10^6 ml)	94 ± 20	12
" " + ILNC (11×10^6/ml)	300 ± 150	39

* ISPC, immune spleen cells.

Test for Lymphotoxin Activity.—The supernatant culture fluid from various control cultures and from cultures of immune LNC with tumor cells were stored according to the method of Granger (7, 23, 24). They were tested for their ability to inhibit protein synthesis of HeLa cells in monolayer culture. The results of this testing are shown in Table II. All supernatants are supplemented with fetal calf serum, antibiotics, and vitamins (7, 23, 24). It can be seen that the super-

natants from ILNC-tumor cell cultures possess lymphotoxin activity, i.e., the ability to inhibit protein synthesis in the tissue culture cells. These supernatant culture fluids, derived from the incubation of 19×10^6 ILNC/ml, and 11×10^6 ILNC/ml, resulted in the synthesis of only 12 and 39% of the control amount of protein-^{14}C by the tissue culture cells. Supernatant fluids obtained from cultures of tumor cells alone, tumor cells plus immune spleen cells, or tumor cells plus NLNC did not result in depressed protein synthesis. The control cultures of NLNC or immune spleen cells did not demonstrate LCT in vitro either.

DISCUSSION

An in vitro model for studying the reaction of immune lymphocytes against BP8 ascites tumor cells and for determining the effect of known enhancing antisera on such interactions has been presented. The results of these experiments indicate that normal LNC or LNC obtained after immunization with Freund's adjuvant do not produce cytotoxicity, whereas specifically immune LNC sensitized in vivo are actively cytotoxic to the tumor cells. Lymphocytotoxic activity as measured in this test is detectable in the lymph node cells only during the period of 5–7 days after immunization, but does not require extremely high lymphocyte:target cell ratios. The ratios used here ranged from 5 to 24:1 with ratios of 10 to 24:1 yielding the most consistent results.

Results with this model have certain implications concerning the effectiveness of different routes of immunization. A doubling of the dose of tumor cells administered intraperitoneally will result in the death of a significant number (approximately 20%) of C57BL mice, yet they are able to tolerate 10^6 cells intraperitoneally or, more notably, 6×10^6 cells when given subcutaneously. This suggests that subcutaneous immunization with its marked stimulation of regional lymphoid tissues is a more potent stimulus to cellular immunity in the lymph nodes tested (axillary, brachial, and inguinal) than is intraperitoneal immunization. In the experiments using lymph node cells after intravenous immunization, a maximum cytotoxicity of 30% was observed with LNC obtained 12–15 days after intravenous immunization. No significant LCT was observed earlier than this. This is in contrast to 100% cytotoxicity obtained with equal or lower concentrations of LNC harvested 5 or 7 days after subcutaneous immunization. These observations also are consistent with the concept of subcutaneous immunization being the most potent route for stimulation of CMI.

The results obtained with the known enhancing antisera in vitro parallel those obtained with the very same sera in vivo (20, 21) and suggest that the in vitro system is, in fact, representative of the in vivo system. Furthermore, it would imply that abrogation of LCT in vitro is dependent upon the same mechanisms and serum factors as enhancement in vivo. Specifically, in both systems antigen-antibody complexes have been shown to be necessary for the mediation of enhancement, while ILNC exposed to antibody alone remain fully active. The

antigen-antibody complexes are able to induce a change in the ILNC which prevents them from attacking fresh untreated tumor cells. It would be of great importance to understand the biochemical alterations in the LNC which have been initiated by the antigen-antibody complexes.

The parallel between events in vivo and those in vitro is further fortified by the data obtained using the virulent tumor which the C57BL mice were unable to reject at the usual dose. In vitro we found a similar event in that LNC obtained after subcutaneous injection with this virulent tumor strain did not express any cytotoxicity.

With regard to the mechanism of antibody-mediated immunosuppression, the results reported here are consistent with a central mechanism of enhancement, i.e., the afferent limb (recognition) of the immune response has taken place in vivo and the effect of the antigen-antibody complexes is on the lymphocyte before it can become an effector cell. The elimination of afferent inhibition as the mechanism of low dose enhancement in vivo already has been suggested by the following observations: (*a*) The quantity of antiserum which will bring about enhancement is minute and would not be expected to cover all the binding sites. (*b*) Even if all the binding sites were covered they would not remain so at 37°C (20). The data obtained here, using cells which have undergone the recognition phase in vivo, add further support to the idea that enhancement must interact in either the central or efferent phase of the immune response.

The experiments in which ILNC are exposed to washed, antibody-treated tumor cells are the strongest evidence for a central mechanism of enhancement. These ILNC when incubated with fresh tumor cells which have never been exposed to antibody are unable to exhibit any cytotoxic activity and ILNC are not affected by incubation with antibody alone. Thus we cannot explain the lack of LCT on the basis of "covering up" of tumor antigen sites. This evidence, along with the above mentioned observations which tend to exclude an afferent mechanism, would indicate that antibody-induced immunosuppression must take place after antigen recognition and before the direct attack of LNC on tumor cells. The effect is one of antigen-antibody complexes on the lymphoid cells themselves and is therefore central.

The mechanism of the cytotoxicity in our assay and in others is not entirely clear. Most workers who have explored this question have indicated a requirement for contact between the cells (25, 26). More recently Granger (7, 23, 24, 27) has developed evidence for the release into the medium of an effector substance, termed lymphotoxin, which causes inhibition of cellular metabolism and eventual cell death.

Testing the supernatants from our system we do, in fact, find evidence for the release of lymphotoxin. The supernatant culture fluid from immune LNC–tumor cell interaction inhibited protein synthesis in the target HeLa cells (Table II). No such inhibition of HeLa cell protein synthesis was obtained with superna-

tants from cultures of tumor cells alone, tumor cells incubated with immune spleen cells, or tumor cells and NLNC.

The relationship of LCT in vitro to lymphocyte transformation and delayed hypersensitivity is not clear. Although Chaparas et al. (28) dissociated DH from lymphocyte transformation using their carbohydrate material, it is not certain whether this can be done with protein antigens as well. Perlmann et al. (29) have separated LCT from lymphocyte transformation, but in our system the BP8 tumor cells can elicit both LCT and stimulation of DNA synthesis in cultured C57BL lymph node cells (30).

With regard to the relationship of lymphocyte transformation and lymphotoxin transformation production, there is preliminary data in this laboratory which suggests that the stimulation of DNA synthesis is neither a necessary nor a sufficient condition for the production of lymphotoxin activity. Although Granger has shown that lymphotoxin production began before there was direct biochemical evidence of stimulation of DNA synthesis, he did not demonstrate that circumstances which lead to lymphotoxin production did not also lead, even if more remotely, to stimulation of DNA synthesis (23).

The relationships of lymphotoxin production, LCT, and lymphocyte transformation remain open to question and are currently under study in this laboratory. It is intriguing to wonder which if any of these processes, passively administered antibody might be capable of shutting off in vivo. Evidence from earlier experiments would suggest that antibody does abrogate LCT in vivo (20). The use of passive antibody to prolong graft survival in humans and efforts to break enhancement in patients with neoplasia will require further knowledge about such basic phenomena.

SUMMARY

The ability of antisera to suppress immune responses either in vivo or in vitro is well known. A variety of lymphocyte-target cell systems have been employed to demonstrate inhibition of cell-mediated immunity by antisera in vitro, and skin, tumor, and kidney graft survival have been prolonged by passively administered antiserum in vivo. An in vitro lymphocyte-tumor cell assay system was developed for the purpose of studying the effects of enhancing antisera (in vivo) on lymphocyte-mediated cytotoxicity in vitro. The characteristics of this system with respect to route of immunization, time of harvest of immune cells, lymphocyte:tumor cell ratio, and effect of nonimmune or nonspecifically immune lymphoid cells are presented. Sera capable of enhancement in vivo were tested in this system and shown to inhibit cell-mediated immunity in vitro. Further, in both instances the immunosuppressive effect is mediated by antigen-antibody complexes and not by free antibody alone.

Experiments were also carried out to determine the site of action of these suppressive antigen-antibody complexes. Presensitized lymphocytes were exposed

to antigen-antibody complexes, washed, and then allowed to interact with fresh tumor cells (not antibody treated). Lymphocytes treated in this manner are incapable of exhibiting cell-mediated immunity in vitro. This evidence supports the concept that the antigen-antibody complexes have a direct immunosuppressive effect on the lymphocyte.

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