EVIDENCE THAT THE L-ASPARAGINASE OF GUINEA PIG SERUM IS RESPONSIBLE FOR ITS ANTILYMPHOMA EFFECTS*,‡

II. Lymphoma 6C3HED Cells Cultured in a Medium Devoid of L-Asparagine Lose Their Susceptibility to the Effects of Guinea Pig Serum in Vivo

By J. D. BROOME,§ M.B.

(From the Department of Pathology, The New York Hospital–Cornell Medical Center, New York)

(Received for publication, April 3, 1963)

The development of insensitivity to guinea pig serum by variant cell lines of Lymphoma 6C3HED has made it possible to examine metabolic features of the lymphoma cells which determine their response, or lack of response, to treatment by guinea pig serum *in vivo*.

Holmquist and Kidd in previous studies from this laboratory showed that such insensitive variants often appeared after treatment of Lymphoma 6C3HED cells *in vivo* with quantities of guinea pig serum insufficient to bring about complete suppression of their growth (1).

The present experiments show that insensitivity to guinea pig serum develops in populations of 6C3HED cells in yet another way, and under conditions in which guinea pig serum is completely absent. It was found that 6C3HED cells could be grown in tissue culture in Eagle's medium. After an initial latent period of 2 or more weeks the cultured cells began to proliferate vigorously, but when these cells were implanted into mice, they were found to produce tumors which were completely and permanently insensitive to guinea pig serum. Eagle's medium contains no L-asparagine. When this amino acid was added to the culture medium not only did it provide a strong growth stimulus for cells freshly placed in culture, but the cells retained their original character of sensitivity to guinea pig serum even after prolonged periods of growth.

These findings are presented in relation to the direct evidence, given in the companion paper, which indicates that the L-asparaginase of guinea pig serum brings about the inhibition of Lymphoma 6C3HED cells *in vivo*.

---

* Support for these investigations was generously provided by Mr. John W. O'Boyle, the American Cancer Society, and The National Cancer Institute.

‡ Partly described in *Nature*, 1961, **191**, 1114.

§ Present address: McEachern Laboratory, University of Alberta, Edmonton, Alberta, Canada.

Materials and Methods

The experiments performed were designed principally to show the relationship between the L-asparagine requirements of 6C3HED cells in tissue culture and their sensitivity or insensitivity to guinea pig serum *in vivo*. They were broadly of 3 kinds. First, by measuring changes in cell number and glucose consumption to trace the developing ability of populations of 6C3HED cells to proliferate in Eagle's medium and with this, after periodically implanting lymphoma cells from the cultures into mice, their loss of sensitivity to guinea pig serum. Secondly, to examine the effect of L-asparagine and other possible growth factors added to the culture medium, in modifying this sequence of changes. Thirdly, to examine the cultural characteristics and L-asparagine requirements of a subline of 6C3HED cells which had previously, under *in vivo* conditions, become insensitive to guinea pig serum.

Mice.—In most experiments ZBC mice of either sex were used, as described in the previous paper. Male C3H mice, obtained from Microbiological Associates, Bethesda, were used in a few experiments as indicated in the text. These mice were kept in the laboratory for a period of at least 1 month before use, and then weighed 22 to 27 gm. 6C3HED tumors grew equally well in both kinds of mice.

Tumor Cells.—6C3HED tumor cells, maintained by subcutaneous transfer in C3H or ZBC mice, as described in the previous paper, were used throughout the present experiments. In most experiments cells of the original guinea pig serum sensitive line were used, but for specific purposes, cells of the guinea pig serum insensitive subline designated 1RG, obtained by the treatment of a 6C3HED tumor *in vivo* with small subcurative quantities of guinea pig serum (1), were used, as will be described. This subline after 4 years of continuous animal passage remained completely insensitive to inhibition by guinea pig serum.

Implantation Techniques.—The techniques used were, with few modifications, those described in the previous paper. Ascites tumors, used in some experiments, were "harvested" from ZBC mice 10 days after the intraperitoneal implantation of 1 million 6C3HED cells obtained from subcutaneous tumors.

The reaction to guinea pig serum of 6C3HED cells grown in tissue culture was tested in the following way: 2 groups of 3 ZBC mice or C3H mice were implanted with 50,000 living tumor cells (counted by the method of Schreck, reference 2) subcutaneously in each flank. Animals in one group acted as untreated controls, animals in the other group were given intraperitoneal injections of 2.0 ml of pooled guinea pig serum (obtained in the manner described in the previous paper) on the day of implantation and on the following 2 days. It will be shown in detail later in the paper that 6C3HED cells maintained for prolonged periods in tissue culture produced tumors *in vivo* with less consistency than cells of the original line carried by repeated transfer in ZBC mice. A test for the reaction of 6C3HED cells to guinea pig serum was considered valid only if each of the 3 control mice developed tumors, and if in these at least 4 of the 6 implanted tumors became palpable. With these provisos, tumor cells were considered to be *sensitive to guinea pig serum* if no palpable tumors developed in the group of treated animals, and all of these remained healthy till the 60th day. Tumor cell sublines were judged to be *insensitive to guinea pig serum*, if using the technique described, no detectable delay occurred in the appearance of tumors in treated animals, compared with untreated controls. Frequently treatment with guinea pig serum caused animals implanted with insensitive cells to develop palpable tumors at an earlier time than in untreated controls. Implantations of tumor cells were judged to be *partially insensitive to guinea pig serum* at an intermediate stage between the two categories described. Here fewer tumors appeared in treated animals, and after a considerably greater latent period than in the controls.

Numerous detailed examples of each of these categories will be given in the text.

Tissue Culture Methods.—Standard techniques of glassware preparation, medium changing, and sterile precautions were used (3).

Culture Medium.—Eagle's medium (4) was used throughout the present experiments. It was prepared from the concentrated stock solutions of salts, glucose, amino acids, and vitamins supplied by Microbiological Associates, to which horse serum was added to make a final concentration of 7 per cent. Medium was prepared freshly at weekly intervals and dispensed into individual flasks for each culture.

In various experiments additional substances were added to the medium. Amino acids, purines, and pyrimidines were obtained from Mann Research Labs, New York, as their analysed grade. Nucleotides and nucleosides were obtained from Schwartz Bioresearch Inc., Mount Vernon, New York. Folic acid and folinic acid were obtained from Sigma Chemical Company, St. Louis. These substances were sterilized before use, usually by passage in solution through a Millipore filter (pore diameter 0.3 micron).

Cultures of 6C3HED cells.—These were prepared using 3 techniques; all were incubated at 37°C.

1. Cultures of cells in serum bottles: In this method, the most widely used in the present experiments, cultures were prepared in tightly stoppered serum bottles of 100 ml capacity. Initially 10 to 20 ml of Eagle's medium was placed in the bottles and ascites tumor obtained from ZBC mice, was added to this in sufficient amount to provide suspensions containing ½ to 1 million living cells/ml.

The culture medium was subsequently changed in accordance with the amount of cell growth and acid production by the cultured cells: between 10 and 20 ml of medium was changed up to 3 times weekly. Fully established cultures were maintained in an actively proliferating state by periodically shaking (1 to 2 times weekly for a vigorous culture) the tumor cells from the bottom of the flask, removing most of the suspension, and replacing it with fresh medium.

2. Cultures of cells in Earle's T flasks: Cultures were inoculated in a similar way to that described using serum bottles. Established cultures grew particularly rapidly in these flasks; the flat bottom allowed microscopical examination of tumor cell morphology and cell counts to be made. In these cultures 10 to 30 ml of fresh medium was added 1 to 3 times per week after pouring off a similar volume of old medium, which inevitably contained a large number of cells. During the course of the present experiments Guerin and Kitchen described their success in culturing 6C3HED cells, by a similar method to this, but using medium M 150 (5).

3. Cultures from solid 6C3HED tumor explants in Carrel flasks: Small portions of solid 6C3HED tumors (approximately 1 mm in diameter) were removed from donor mice under sterile conditions and placed in Carrel flasks on a layer of bovine embryo extract freshly mixed with chicken plasma. A coagulum from the chicken plasma rapidly formed and this, containing the tumor tissue, was covered with 10 ml of Eagle's medium. Initially the outgrowth from the explant consisted largely of fibroblast-like cells, but tumor cells subsequently grew more actively, as will be described later. To these cultures 10 ml of fresh medium was added 1 to 3 times per week after the old medium had been poured off.

Quantitation of Cell Growth.—

1. In Earle's T flasks: After allowing the cultured cells to settle on the flat bottom of the flask, the number of cells present in 100 fields was counted using a Zeiss inverted microscope with phase-contrast optics and × 25 objective. By measuring both the area of the microscopic field with a ruled slide and the total surface area of the flask bottom, it was possible to calculate the total number of cells in the culture.

2. In subcultures from serum-bottle cultures: Subcultures of cells in 3 ml of fresh medium were made in Pyrex test tubes, containing small teflon-coated magnetic stirrers. Cell counts were performed after diluting the suspensions with an equal volume of 1 per cent trypan blue in Ringer's solution and mixing carefully. Counts were adjusted in most experiments to be-

tween 170,000 and 230,000 living cells per ml, the final count being accurately measured. After bringing the pH to 7.0, the tubes were tightly stoppered and incubated for 48 hours at 37°C. At the end of the incubation period cells in the subcultures were suspended by the magnetic stirrer, and their number in samples removed was counted again after dilution in 1 per cent trypan blue.

Glucose Estimations.—Glucose was estimated in samples of culture medium by the anthrone method (6).

RESULTS

The Behavior of 6C3HED Cells in Tissue Culture in Eagle's Medium.—When cultures of solid explants of 6C3HED tumors were prepared in Eagle's medium, they underwent a very characteristic sequence of changes in their growth and metabolic activity. For the first 2 to 3 days small amounts of acid metabolites were produced, as shown by the change in color of the methyl red indicator in the medium, but after this time acid production decreased sharply and in the following weeks was almost absent. Observation during this time showed that although a considerable proliferation of fibroblast-like cells was taking place at the edge of the explant, lymphoma cells remained confined to the central mass without any apparent growth. After a period of 2 to 5 weeks, however, a remarkable change occurred, the lymphoma cells began to proliferate vigorously, spreading along the bottom of the flask, and rapidly becoming the dominant cell in the culture. When the medium was disturbed it became turbid from suspended cells; acid production and glucose consumption increased markedly.

A similar sequence of events was observed in cultures made from ascitic fluid and grown in flat bottomed T flasks. Again, an initial proliferation of fibroblast-like cells occurred, but with this the lymphoma cell population declined remarkably, as shown in Fig. 1. Here, the culture initially contained 30 million tumor cells, but after 14 days, counts showed that less than 100 thousand remained. This small number of cells did not increase significantly for a further 3 weeks, but then a slow increase was observed (to 300 thousand at 5 weeks) followed after a further week by a very rapid rise to 10 million.

Attempts to determine changes in the number of cells in serum bottle cultures were unsuccessful; the disturbance involved in repeatedly suspending the cultured cells for sampling invariably resulted in their death. However, changes in the metabolic activity of the cultures could be clearly demonstrated by following their glucose consumption, as may be seen in Fig. 2. In this the 2 cultures used were maintained using different techniques but showed essentially similar features. Culture 1 showed a moderate glucose utilization in the first 2 days (average 9.9 mg/day) and subsequently a decline to a level of 4.1 to 7.0 mg/day until the 19th day. After this time a sharp rise in glucose consumption occurred. On the 21st day the consumption was 12.7 mg/day, by the 24th, 16.2 mg/day, and afterwards it reached a plateau level. Culture

2 showed similar changes but the maintenance technique did not permit such vigorous proliferation as in culture 1. The initial glucose consumption, 1.1 mg/day, was extremely low but rose at the 4th day to 3.6 mg/day and continued at a low level, declining to 2.8 mg/day by the 19th day. At this time again a sharp increase in utilization occurred, reaching 7.2 mg/day by the 21st day and afterwards continuing at this raised level.

The latent interval, before vigorous growth of cultures occurred, varied considerably in different experiments despite apparently similar conditions

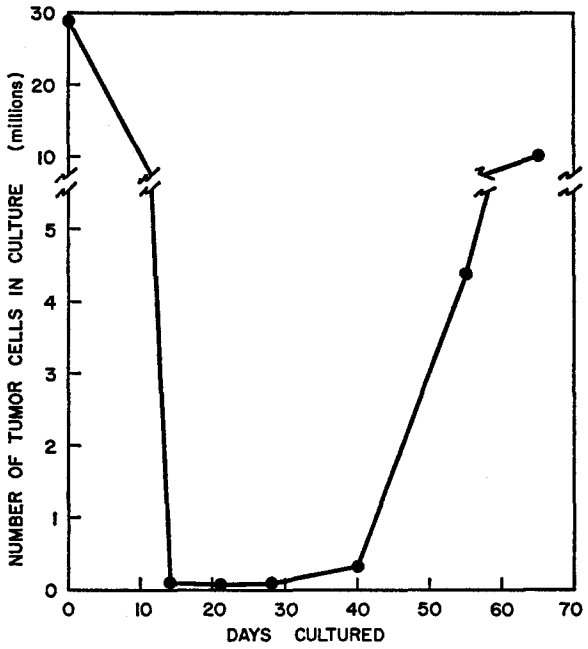


FIG. 1. Changes in the number of 6C3HED cells in a culture made in Eagle's medium.

under which the tumor cells were maintained. Thus in 10 cultures made on different occasions, in which the duration of latency is recorded, vigorous growth began in the 2nd week in 2 cultures, in the 3rd week in 4 cultures and in the 4th week in a further 4 cultures. But by using careful technique, its occurrence was invariable; 27 cultures were grown in unmodified Eagle's medium in this way.

Subcultures of tumor cells could readily be made once the change to vigorous growth had occurred. The original cultures were gently agitated to suspend part of the tumor cell sediment but to leave cells of other kinds attached to the glass. A small quantity of suspension was then used to inoculate fresh bottles of

medium. Cells from 2 cultures were transferred on 5 successive occasions, over a period of 8 weeks, into fresh culture bottles and grew vigorously throughout this time. 5 other cultures transferred on 3 occasions over a similar time again continued vigorous growth.

6C3HED cells whether obtained from solid subcutaneous tumors or from ascites tumors, had the typical appearance of lymphoblasts. When smears were prepared and stained by the May-Grunwald–Giemsa method, the cells were

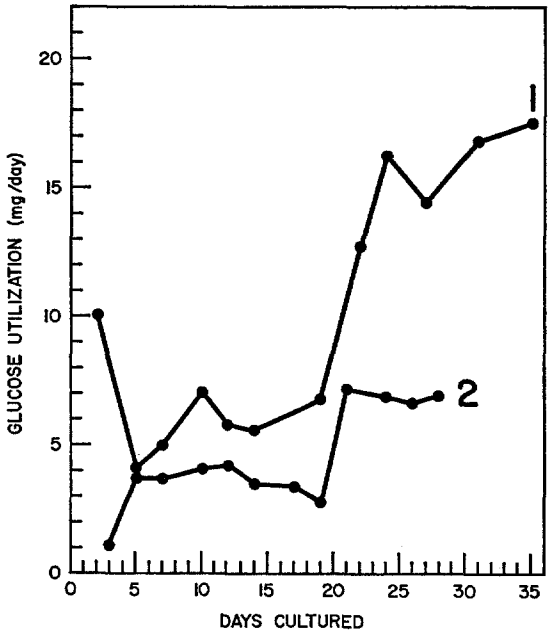


FIG. 2. Glucose consumption of 2 cultures of 6C3HED cells in Eagle's medium.

In both experiments cells were grown in serum bottles. In culture 1 sufficient medium was added 3 to 4 times per week to maintain the pH of the medium at 7.0 — 7.2. In culture 2, the medium was completely changed on each occasion; this technique was obviously less satisfactory for obtaining maximum cell proliferation.

seen to have a rounded surface enclosing a thin rim of cytoplasm and a large oval or round nucleus. Nuclei showed a delicately latticed chromatin pattern and often prominent nucleoli. There was considerable variation in cell size, from small cells of 14 microns to others of 35 microns or more in diameter; giant cells with multiple and bizarre nuclei were occasionally seen. The appearance of cells grown in culture even for prolonged periods was very similar to that just described but for still greater variation in cell size and an increasing frequency of abnormal giant cell forms.

The cultured cells retained their ability to produce lymphomas when implanted into mice.

Tumor Growth in Vivo Resulting from the Implantation of 6C3HED Cells Grown in Tissue Culture.—6C3HED cells grown in culture for periods of up to 8 weeks, and by this time proliferating vigorously, produced tumors in ZBC or C3H mice in the same way as did cells maintained by serial animal passage. Palpable tumors regularly appeared on the 9th to 11th days following the implantation of 50,000 living tumor cells subcutaneously in each flank and these grew progressively until the death of the animals on the 25th to 35th days.

TABLE I

Results of Implanting Cells of Lymphoma 6C3HED in Vivo after Culture in Eagle's Medium for Varying Periods

Experiment	Period of culture	No. of lymphoma cell implantations	Outcome of implantations*	
			No. of palpable tumors produced	No. of tumors regressing
	wks.			
Various	2–5	6‡	6	0
GRS 71	8	6	6	0
TC 17	9	12	12	2
TC 10	12	6	6	0
GRS 95A	15	6	4	0
GRS 91	19	6	4	0
TC 13	21	12	12	0
TC 10	24	12	11	2
TC 17	29	6	6	4
TC 10	32	6	5	2

* 3 or 6 ZBC mice in each test were implanted with 50,000 living lymphoma cells in each flank subcutaneously. Mice were examined for tumor growth not less frequently than on alternate days from the 9th to 28th day after implantation, and from this time twice weekly until death, or in the case of animals not having tumors, till the 60th day.

‡ In each of 10 different experiments.

But cells from cultures maintained for periods of more than 8 weeks produced increasingly erratic results. At some implantation sites (see Table I) tumors entirely failed to appear, and at others the latent period of the tumors was prolonged, to as much as 20 days in the case of implantations from 1 culture grown for 19 weeks. Further, a proportion of the tumors, after becoming palpable, underwent spontaneous regression. This rarely occurs amongst tumors maintained by *in vivo* passage, and has only been observed on 5 occasions in more than 4000 ZBC mice implanted over the course of 3½ years with 5000 to 50,000 6C3HED cells on each side. Table I shows the increasing frequency with which regression occurred in tumors produced by cultured cells. In TC 17, grown for 29 weeks in culture, 4 out of 6 tumors eventually regressed.

Other properties of the 6C3HED cells were altered by culture as will now be shown.

The Effect of Guinea Pig Serum on 6C3HED Cells Grown in Culture and Subsequently Implanted into ZBC Mice.—Now, as earlier studies from this laboratory have shown, 6C3HED cells, immediately after implantation into mice, are extremely sensitive to inhibition by guinea pig serum. Thus, in mice implanted with 50,000 cells in each flank, a single injection of 0.25 ml guinea pig serum regularly produced a detectable delay in the time at which the tumors became palpable (see for example Table II of the previous paper) 1 ml of guinea pig serum produced the complete suppression of tumor growth in 30 to 100 per cent of treated mice, and 2.0 ml with rare exceptions cured all the treated animals.

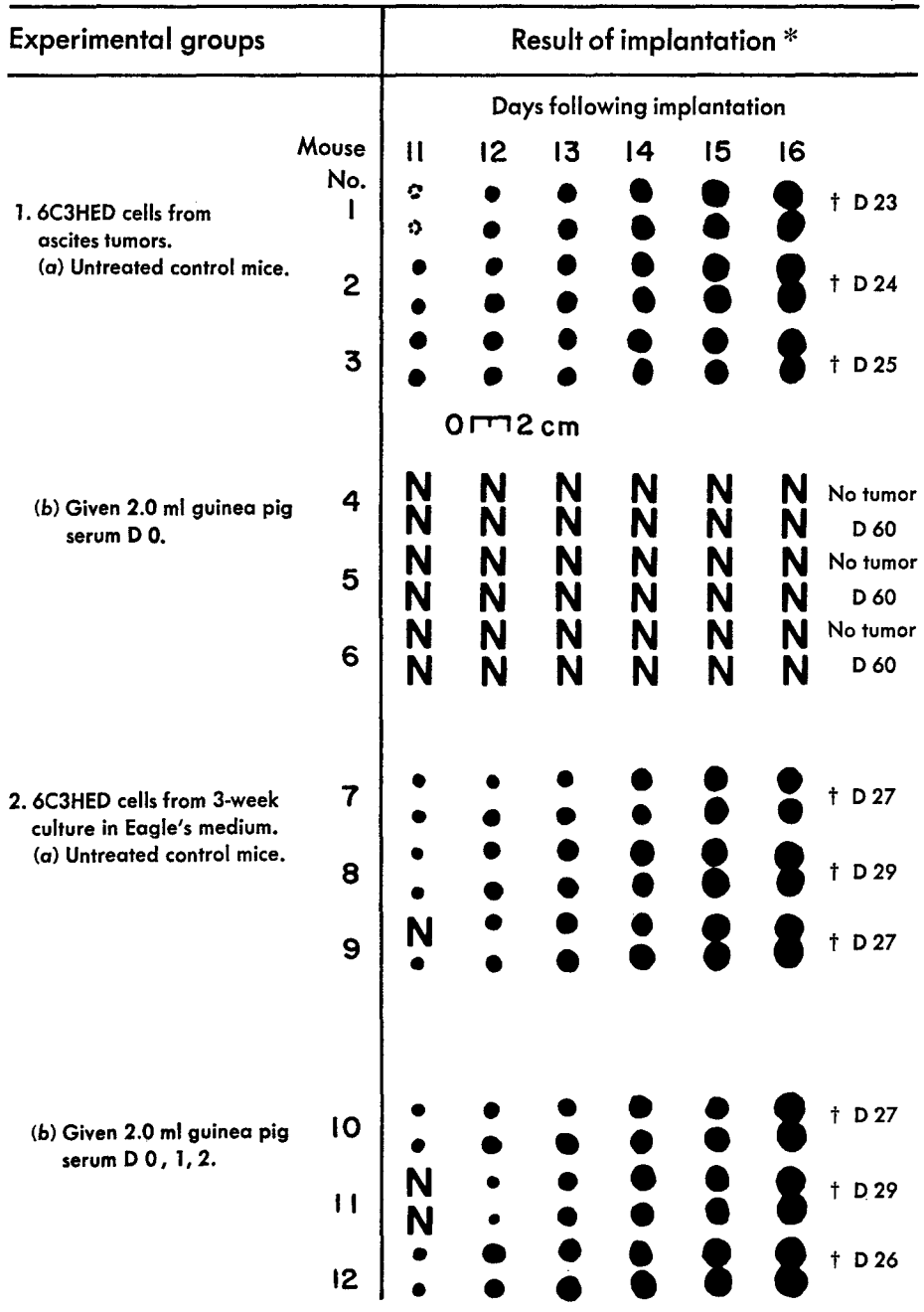
But when 6C3HED cells, which had begun to proliferate vigorously on culture in Eagle's medium, were implanted into mice, treatment with guinea pig serum produced no inhibition whatsoever. Even injections of 2.0 ml of pooled guinea pig serum given immediately following implantation and on each of the 2 successive days produced no inhibitory effect, as is shown in Fig. 3. Indeed, in other experiments such treatment often resulted in an acceleration of tumor growth, tumors appearing in treated animals 1 to 2 days before those of the controls.

Cells cultured from solid explants of 6C3HED tumors in Eagle's medium similarly became insensitive to guinea pig serum. In the experiment illustrated in Table II a number of cultures were begun simultaneously, 1 from cells of ascites tumor and 6 from solid explants. Cells from the ascites tumor after 3 weeks in culture were completely insensitive to guinea pig serum. In this and other experiments there was an intermediate stage before complete insensitivity to guinea pig serum was demonstrated. After 2 weeks incubation in tissue culture, cells implanted into mice and tested with guinea pig serum were not completely suppressed, as they had been 1 week earlier; tumors grew out from 2 of the 6 implantations but after latent periods prolonged to 17 and 20 days. This stage of "partial insensitivity" is probably due to the cultured cells consisting of a mixed population of sensitive and insensitive cells with the latter in relatively small number.

Cells from a culture of solid explant tested after 2 weeks' incubation were partially insensitive to guinea pig serum, although 2 other cultures tested after 3 and 4 weeks' incubation remained sensitive. But on testing cultures maintained for 7 and 12 weeks the cells in both were completely insensitive to guinea pig serum.

A more detailed examination of the time taken for the loss of guinea pig serum sensitivity in cultures of ascites tumor cells is shown in Table III.

It will be seen that all samples of ascites tumor cells used for inoculation of the cultures were fully sensitive to guinea pig serum, and so too cells maintained in culture for 4 days; in 4 7-day cultures only 1 showed any degree of guinea pig



D = day

* ZBC mice implanted subcutaneously with 50,000 6C3HED cells in each side.

FIG. 3. The development of insensitivity to guinea pig serum by 6C3HED cells cultured in Eagle's medium.

serum insensitivity. At 14 days however, cells from 1 of 4 cultures tested showed complete insensitivity, cells from another showed partial insensitivity

TABLE II

The Development of Insensitivity to Guinea Pig Serum by 6C3HED Cells in Tissue Cultures Grown from an Ascites Tumor and from Solid Explants

Culture No.	Cultures implanted with 6C3HED cells derived from:	Period of culture	Reaction of cultured cells to guinea pig serum after implantation *in vivo*
		wks.	
1	Ascites tumor	1	Sensitive
		2	Partially insensitive*
		3	Completely insensitive
		4	Completely insensitive
2	Solid explant	1	Sensitive
3		2	Partially insensitive‡
4		3	Sensitive
5		4	Sensitive
6		7	Completely insensitive
7		12	Completely insensitive

6C3HED cells, from a suspension shown on testing to be fully sensitive to guinea pig serum *in vivo*, were implanted into a group of ZBC mice, each animal receiving 1 million cells intraperitoneally and 500 thousand cells subcutaneously in both flanks. 10 days later 1 of the mice was killed; ascitic fluid was removed and inoculated into a serum bottle containing Eagle's medium to produce a suspension containing 900 thousand living tumor cells/ml. Portions of the subcutaneous tumors were also removed and cultured in the way described in the section entitled Materials and Methods. At intervals shown in the table samples were removed from the serum bottle cultures and 50,000 living cells were implanted in each flank of 6 ZBC mice; 3 of these were used as untreated controls, 3 were given injections of 2 ml of guinea pig serum on the day of implantation and the 2 succeeding days. Similarly, cultures of the solid tumor explants in Carrel flasks were broken up as finely as possible with a platinum loop and suspended in culture medium. No attempt was made to measure the number of lymphoma cells in these suspensions. They were injected in 0.5 ml volumes subcutaneously into ZBC mice and the reaction of the lymphoma cells to guinea pig was tested as described above.

* 2 out of 6 implanted tumors appeared in animals treated with guinea pig serum, these after latent periods of 17 and 20 days. All 6 implanted tumors grew out in untreated controls on the 10th and 11th day.

‡ 3 out of 6 implanted tumors appeared in animals treated with guinea pig serum, after latent periods of 16 to 20 days. All implanted tumors grew out in untreated controls on the 13th and 16th day.

while 2 remained fully sensitive to treatment. At 21 days cells from all 4 cultures tested were insensitive to guinea pig serum, but 1 culture tested at 28 days remained fully sensitive. This is the longest period of culture under these conditions in which 6C3HED cells maintained their sensitivity to guinea pig serum.

This culture was unfortunately not tested at a later time. Cells from 4 cultures tested at 28 days and 5 at 35 days were completely insensitive.

The Persistence of Guinea Pig Serum Insensitivity Developed by 6C3HED Cells in Tissue Culture.—Cells of Lymphoma 6C3HED maintained in culture for periods of 2 to 10 weeks, during which time they became insensitive to guinea pig serum, maintained the character on growth *in vivo*. As may be seen from Table IV tumor cells even after as many as 14 serial transfers in ZBC mice, which were implanted with a large number of cells on each occasion (2 to 5 million) remained completely insensitive to guinea pig serum.

TABLE III

The Development of Insensitivity to Guinea Pig Serum by 6C3HED Cells Cultured in Eagle's Medium

Period in culture	Total No. of tests	Results of treating 6C3HED cells implanted in ZBC mice with guinea pig serum		
		Cells sensitive to guinea pig serum	Cells partially insensitive to guinea pig serum	Cells completely insensitive to guinea pig serum‡
days				
0*	17	17	0	0
4	4	4	0	0
7	4	3	1	0
14–17	4	2	1	1
21	4	0	0	4
28	5	1	0	4
35	5	0	0	5

* Lymphoma cells tested were those obtained from a number of ascites tumors used for inoculation of tissue cultures.

‡ Results shown are those of the first demonstration of guinea pig serum insensitivity in each culture.

Cells from some 18 cultures were retested with guinea pig serum after the development of insensitivity; 2 of these cultures had been grown for total periods of 29 and 32 weeks respectively, 2 more grown in the original bottles for 10 and 11 weeks had been subcultured on 6 successive occasions. After these prolonged periods, the cultured cells remained completely insensitive to guinea pig serum.

Two changes in the characteristics of 6C3HED in tissue culture have therefore been observed, first the acquisition of the ability to proliferate in Eagle's medium, secondly the permanent loss of sensitivity to guinea pig serum. The important question therefore arises of whether both changes are expressions of the same underlying alteration or alterations in the tumor cell. Both characteristics appear to change at the same time. When cells of 10 cultures maintained

for 3 to 5 weeks and tested within 1 week of the beginning of obvious *in vitro* proliferation were implanted into mice, all were either partially or completely insensitive to guinea pig serum. However, in 5 other experiments in which the period of culture varied from 2 to 5 weeks, insensitivity to guinea pig serum was demonstrated in cells from cultures which were not yet showing active proliferation. But in each of these despite the disturbance of removing cells for implantation, proliferation became obvious a few days later.

A consideration of the results of these and other experiments which have been described suggested the possibility that the changed characteristics of 6C3HED cells in tissue culture might be related to adaptation to growth in the absence of a normally essential metabolite. The experiments described in the previous

TABLE IV

Insensitivity to Guinea Pig Serum Developed by 6C3HED Cells in Tissue Culture Persisting on Subsequent Growth in Vivo

Culture	Period in culture*	No. of cells used for *in vivo* transfers‡	Complete insensitivity to demonstrated after the number of transfers shown
	wks.		
1	10	50 thousand	8
2	2	50 thousand	6
3	5	2 to 5 million	11
4	3	2 to 5 million	14

* Cultures maintained in serum bottles containing Eagle's medium.

‡ Transfers of subcutaneous tumors in ZBC mice were performed at intervals of 10 to 14 days. Each flank was implanted with the number of cells shown in the table.

paper, showed that L-asparaginase possessed many of the properties of the tumor inhibitory agent in whole guinea pig serum. Knowing that L-asparagine was absent from unmodified Eagle's medium, experiments were undertaken first to study its effects when added to Eagle's medium on the growth of 6C3HED cells, and secondly, to determine whether 6C3HED cells grown in the presence of this amino acid retained their sensitivity to guinea pig serum.

Effects of L-Asparagine on the Growth of 6C3HED Cells in Tissue Culture.— When L-asparagine was added to Eagle's medium in which 6C3HED cells were cultured a striking difference was observed in their activity. For instead of the initial period of 2 to 4 weeks during which proliferation of the cultured cells failed to occur, cells in Eagle's medium containing 20.0 to 200.0 mg L-asparagine/liter grew profusely from the beginning.

Fig. 4 shows the reflection of this in the glucose consumption of 2 cultures. The first culture contained no added L-asparagine, and as described previously, after the first 2 days its glucose utilization dropped to a low level (4.1 to 7.0

mg/day) until on the 22nd day it was observed to have sharply increased (to 12.6 mg/day). By contrast, in the second culture, containing 200.0 mg L-asparagine/liter, glucose consumption rose continuously from the beginning, with the exception of a minor inconsistency found in samples of medium examined on the 12th and 14th day. In the first 2 days the glucose consumption was 9.0 mg/day, on the 9th day it was 14.4 mg/day, on the 16th day 15.5 mg/day, and on the 27th day 20.4 mg/day. In 3 other cultures prepared by slightly differing

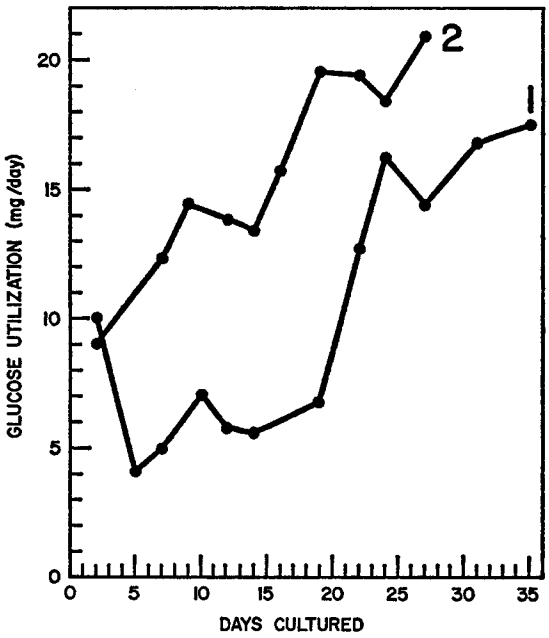


FIG. 4. Glucose consumption of cultures of 6C3HED cells in unmodified Eagle's medium and in Eagle's medium containing added L-asparagine. Culture 1, unmodified Eagle's medium. Culture 2, Eagle's medium + L-asparagine 200 mg/liter.

Cultures initially contained 10 million cells obtained from ascites tumors, in 20 ml medium. The medium was partially changed 1 to 4 times each week to maintain the pH at 7.0 — 7.2.

experimental techniques, glucose consumption of the cells cultured in media containing from 20.0 to 200.0 mg/liter L-asparagine showed a similar pattern of vigorous growth from the start.

A growth stimulus was also shown in subcultures of 6C3HED cells made in modifications of Eagle's medium containing varying concentrations of added L-asparagine. Fig. 5 shows the results from 3 experiments in which the cells used were taken from serum bottle cultures inoculated 2 to 3 weeks previously.

A number of features are clear from the graphs: in all 3 experiments the cell count decreased in subcultures grown in unmodified Eagle's medium, in Ex-

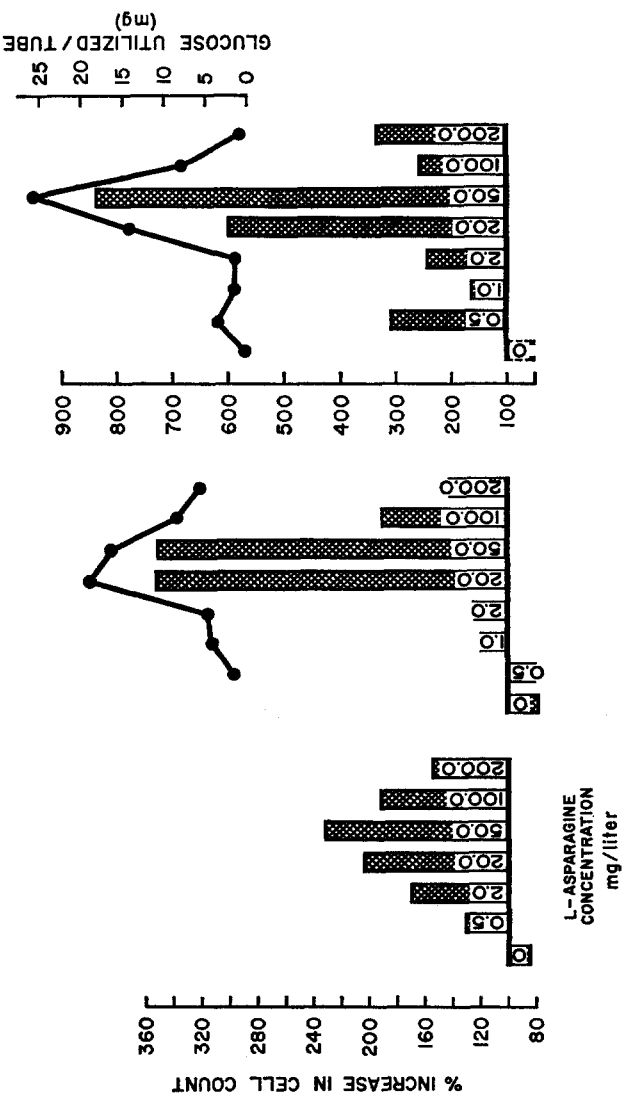


FIG. 5. The effect of different concentrations of L-asparagine on the growth rate and glucose utilization of 6C3HED cells in Eagle's medium, after 2 to 3 weeks *in vitro*.

Experiment *A*. Subcultures containing 170,000 to 200,000 living cells/ml were prepared from cultures initially grown for 14 days. The cells in the subcultures were recounted after an incubation period of 42 hours.

Experiment *B*. Subcultures containing 170,000 to 200,000 living cells/ml were prepared from cultures initially grown for 14 days. The cells in the subcultures were recounted after an incubation period of 48 hours.

Experiment *C*. Subcultures containing 70,000 to 90,000 living cells/ml were prepared from cultures initially grown for 21 days. The cells in the subcultures were recounted after an incubation period of 120 hours.

periment *A* to 84 per cent of the number of implanted cells, and in Experiment *B* to 78 per cent. In Experiment *C* no living cells could be found at the end of the period of incubation. L-Asparagine, even in concentrations of 0.5 and 1.0 mg/liter produced a detectable growth stimulus. The optimal concentration in 2 experiments was 50.0 mg/liter, in 1 20.0 mg/liter. The highest concentrations of L-asparagine used, 100.0 and 200.0 mg/liter provided a less effective growth stimulus.

In Experiments *B* and *C* the amounts of glucose consumed by the cultured cells were estimated and closely followed the rate of growth as shown by the cell counts.

In Experiments *A* and *B* in which tumor cells before subculturing were grown for 14 days in the concentrations of L-asparagine shown, the cells, on implantation into ZBC mice at the end of the experiment had all retained their sensitivity to guinea pig serum. In both experiments concentrations of L-asparagine from 0.5 to 2.0 mg/liter provided an increasingly effective growth stimulus. In Experiment *C* however, the tumor cells were initially grown for 21 days before subculturing, and here cells in the subculture containing 0.5 mg of L-asparagine/liter grew more rapidly (to 307 per cent of the initial count) than those containing 1.0 mg of L-asparagine/liter or 2.0 mg of L-asparagine/liter. But on implantation into ZBC mice, cells from the subculture containing 0.5 mg of L-asparagine/liter were completely insensitive to guinea pig serum, those in the subculture containing 1.0 mg/liter were partially insensitive, and those in the subculture containing 2.0 mg/liter were fully sensitive. Thus as described earlier, the ability of 6C3HED cells to grow in low concentrations of L-asparagine was closely related to their developing insensitivity to guinea pig serum.

After 9 weeks' growth, cells were taken from the cultures used in Experiments *B* and *C*, and fresh subcultures were made. As may be seen in Fig. 6, the growth pattern was entirely different from that found previously. In cultures containing no L-asparagine the number of cells increased in the 2 experiments to 188 and 248 per cent respectively of the initial counts. Cultures in media containing 0.5 to 2.0 mg of L-asparagine/liter also multiplied considerably more rapidly than before. Better growth in tissue culture also occurred in cultures containing 20.0 to 200.0 mg of L-asparagine/liter, and was especially marked in cultures containing 50 mg of L-asparagine/liter. The growth rates of 773 and 750 per cent which occurred in 2 subcultures were 2 to 3 times as great as those found when the cultures had been first examined 7 weeks previously.

The Effect of L-Asparagine Added to Eagle's Medium on the Development of Insensitivity to Guinea Pig Serum by 6C3HED Cells.—The experiments described earlier showed that 6C3HED cells cultured in unmodified Eagle's medium invariably became insensitive to guinea pig serum within a period of 5 weeks. In the presence of sufficient L-asparagine no such change occurred.

0.5 mg L-asparagine/liter, which produced no more than an equivocal growth

stimulus, failed to have any detectable effect on the development of insensitivity to guinea pig serum, as is shown in Table V, and 1.0 mg/liter was also ineffective. A slight delay in the development of guinea pig serum insensitivity was produced in 3 out of 4 experiments by a concentration of L-asparagine of 2.0 mg/liter, compared with control cultures in unmodified medium, but on prolonging the period of culture to 4 to 6 weeks, the development of guinea pig serum insensitivity always occurred. Concentrations of 20.0 to 200.0 mg of

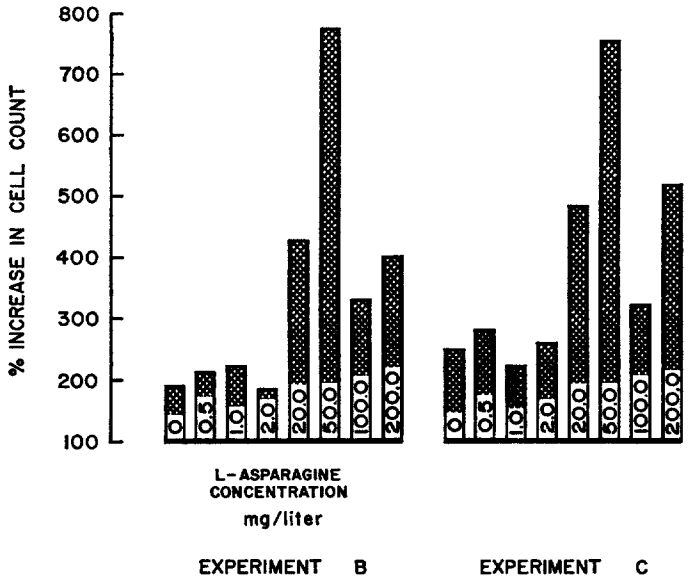


FIG. 6. The effect of different concentrations of L-asparagine on the growth rate of 6C3HED cells in Eagle's medium, after 9 weeks *in vitro*.

Subcultures initially contained 170,000 to 200,000 living cells/ml. Cell counts were repeated after 48 hours incubation.

L-asparagine/liter however, in all cases prevented this change, even in cultures grown for periods of up to 29 weeks, as shown in Table VI.

Although most cultures were maintained during the whole experimental period without transfer to fresh culture bottles, active proliferation occurred throughout this time. Accumulations of cells on the bottom of the culture bottles were poured off, usually at weekly intervals; counts showed that 20 to 40 million cells were removed on each of these occasions. The most rapid multiplication occurred in Experiment 6 shown in Table VI, in which 6C3HED cells were grown in an Earle's T flask. 3 times each week, the medium and suspended cells were poured off before replacement with fresh medium. On each occasion 20 to 30 million cells were removed.

Cells from 2 cultures were grown in serum bottles containing Eagle's medium to which 50.0 mg L-asparagine/liter had been added. After 10 and 12 weeks respectively these cells were subcultured by 5 successive transfers over a period of 8 weeks. On implantation into ZBC mice the lymphoma cells remained perfectly sensitive to inhibition by guinea pig serum.

A further test was performed to exclude the possibility that "storage" of L-asparagine or another temporary change occurring in lymphoma cells cultured in media with added L-asparagine, was masking the demonstration of guinea pig serum insensitivity when these cells were implanted *in vivo*. Cells were obtained from 3 cultures, grown for 9 to 14 weeks, 2 with 50.0 mg of L-asparagine/liter and 1 with 200.0 mg/liter. These cells were implanted into groups of ZBC mice, and 10 to 15 days later cells were removed from the tumors which

TABLE V

The Effect of Different Concentrations of L-Asparagine in Preventing the Development of Guinea Pig Serum Insensitivity by 6C3HED Cells in Culture

Experiment	Period of culture	Reactions to guinea pig serum of 6C3HED cells cultured in Eagle's medium containing the following concentrations of added L-asparagine, *mg/liter*							
		0	0.5	1.0	2.0	20.0	50.0	100.0	200.0
	wks.								
1	3	—	I	I	PI	S	—	—	—
	6	—	—	I	I	S	—	—	—
2	3½	I	I	I	I	S	S	S	S
3	3	—	I	PI	S	S	S	S	S
4	4	—	I	I	PI	S	S	S	S

S, sensitive; PI, partially insensitive; I, completely insensitive.

developed and reimplanted into further groups of animals. The tumor cells at this time remained fully sensitive to guinea pig serum.

Since L-asparagine added to Eagle's medium prevented the development of guinea pig serum insensitivity by 6C3HED cells in culture, experiments were performed to determine whether cultures of cells which had become insensitive in the absence of the amino acid would revert to the guinea pig serum sensitive state on further culture in media containing concentrations of L-asparagine which produced rapid growth.

In 2 experiments cells which had gained the character of guinea pig serum insensitivity during growth for 5 and 28 weeks respectively in unmodified Eagle's medium were subcultured into media containing L-asparagine in concentrations of 50.0 and 200.0 mg/liter. But on testing cells from the subcultures 8 weeks later, they remained completely insensitive to guinea pig serum. A similar failure to obtain reversion to guinea pig serum sensitivity occurred in

another experiment which was performed with the aim of providing a growth advantage for cells which could utilize L-asparagine in place of L-glutamine. Cells initially grown for 29 weeks in Eagle's medium were subcultured into a medium in which the concentration of L-glutamine was progressively lowered, from an initial level of 300.0 mg/liter, and replaced with an equal amount of

TABLE VI

The Prevention of the Development of Insensitivity to Guinea Pig Serum in 6C3HED Cells Grown in Tissue Culture by the Addition of L-Asparagine to the Medium

Experiment No.	Sensitivity or insensitivity of 6C3HED cells grown in tissue culture to inhibition by guinea pig serum *in vivo*		
	Cultures containing no added L-asparagine	Cultures containing added L-asparagine	
	Cells cultured for the following periods found to be *insensitive*	Concentration of L-asparagine	Cells cultured for the following periods found to be *sensitive*
	wks.	*mg/liter*	*wks.*
1	2½	200	7
2	2	200	12
	3	200	11
		20	11
3	3	20	24
		50	24
		100	24
		200	24
4	3	20	21
		50	21
		100	21
		200	21
5	Not tested	50	29
6	Not tested	20	22

L-asparagine. Most of the cultured cells died when the concentration of L-glutamine was decreased below 100.0 mg/liter, but the survivors began to proliferate on changing the composition of the medium to 100.0 mg of L-glutamine and 200.0 mg of L-asparagine/liter. After growing the cells in this medium for 12 weeks they were implanted into mice, but they remained completely insensitive to guinea pig serum.

To test further the relationship between the sensitivity of 6C3HED cells to guinea pig serum and their dependence in tissue culture on an external source

of L-asparagine, cultures were prepared of a subline of 6C3HED cells which had become insensitive to guinea pig serum *in vivo*.

Growth Properties in Tissue Culture of 6C3HED Cells Which under in Vivo Conditions Had Become Insensitive to Guinea Pig Serum.—By treating 6C3HED lymphomas growing *in vivo* with sublethal quantities of guinea pig serum, Holmquist and Kidd produced a number of sublines which on further treatment with guinea pig serum were completely and permanently insensitive to its inhibitory effects (1). Tissue cultures were prepared from cells of one of

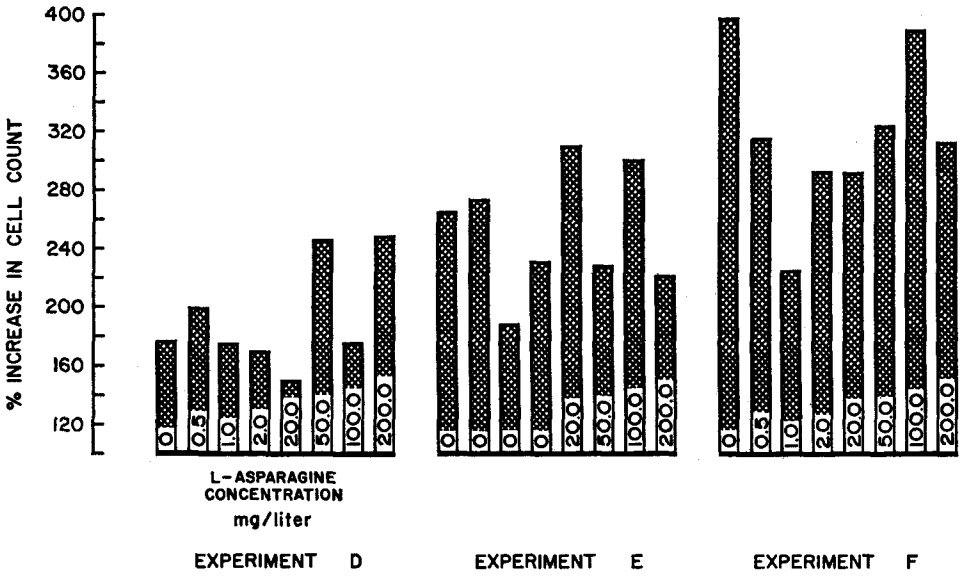


FIG. 7. The effect of different concentrations of L-asparagine on the growth rate of 6 C3HED cells of the guinea pig serum insusceptible subline 1RG, after 2 weeks *in vitro*.

Subcultures initially contained 170,000 to 200,000 living cells/ml. Cell counts were repeated after 48 hours incubation.

these sublines, designated 1RG, and their L-asparagine requirements were examined. Cultures of 1RG cells in unmodified Eagle's medium grew vigorously, without showing any initial lag period comparable to that which occurred using cells of the original guinea pig serum sensitive line. In a serum bottle culture containing an initial inoculum of 30 million cells, in which glucose utilization was examined, a high rate of utilization (11.5 to 16.0 mg/day) was maintained throughout the first 14 days of culture. So too, 1RG cells taken from cultures incubated for 2 weeks and subcultured in the way described in the previous section, multiplied very vigorously in the complete absence of L-asparagine, as may be seen in Fig. 7. In 1 of the 3 experiments shown, *F*, the subculture con-

taining no L-asparagine multiplied in a 48 hour period to 400 per cent of the initial count, an increase greater than that occurring in any other subculture, even though these contained amounts from 0.5 to 200.0 mg/liter of L-asparagine. In 2 other experiments, *D* and *E*, the medium containing no asparagine similarly permitted vigorous growth of the tumor cells, but cells in media containing 20.0 to 200.0 mg of L-asparagine/liter multiplied usually slightly more rapidly.

In the way described in the previous section, attempts were made to see whether 1RG cells would become sensitive to guinea pig serum on prolonged culture in media containing L-asparagine. In 3 experiments in which cells were incubated for 11, 17, and 19 weeks respectively in medium containing 200.0 mg/liter of L-asparagine, the cells remained completely insensitive to guinea pig serum, and so too when cells were cultured in the minimum concentration of L-glutamine (50.0 mg/liter) which permitted growth, with a high concentration of L-asparagine (250.0 mg/liter) the procedure failed to produce any sign of guinea pig serum sensitivity in the lymphoma cells.

Experiments Designed to Elucidate the Role of L-Asparagine in the Metabolism of 6C3HED Cells.—The experiments which have been described have shown that 6C3HED cells sensitive to guinea pig serum require L-asparagine for growth in tissue culture; they have not however provided any evidence on the function of this amino acid in tumor cell metabolism. L-Asparagine is present in most if not in all animal proteins and therefore must at least be required by 6C3HED cells for protein synthesis, but the additional possibility that the amino acid and its amide group might be required for other metabolic processes, was tested in a number of experiments.

In these, test substances were incorporated into tissue culture media to determine whether they could act as substitutes for L-asparagine to permit active growth of the lymphoma cells and to prevent the development of guinea pig insensitivity.

None of the 16 amino acids which were tested was able to substitute for L-asparagine as is shown by the experiments summarized in Table VII. D-Asparagine was unable to substitute for its stereoisomer, and L-aspartic acid was also without significant effect.

The effects of L-aspartic acid incorporated in Eagle's medium on the characteristics of 6C3HED cells in culture was tested in 2 experiments. In the 1st experiment cells were cultured in a concentration 200.0 mg of L-aspartic acid/liter, but initially they grew very poorly. In the control culture containing unmodified Eagle's medium the lymphoma cells began to proliferate after 2 weeks' incubation and on testing 1 week later they were completely insensitive to guinea pig serum. At this time however it was not possible to obtain sufficient living cells from the culture containing L-aspartic acid to test their reaction to guinea pig serum. But after five weeks' incubation active proliferation began

and the lymphoma cells were then found to have become guinea pig serum insensitive.

Another experiment provided a suggestion that L-aspartic acid might have some L-asparagine sparing effect. In this a series of cultures were prepared in serum bottles containing Eagle's medium modified by the addition of 1.0 mg of L-asparagine/liter (a quantity which had been repeatedly shown to produce a mild growth stimulus for the lymphoma cells but which was insufficient to prevent development of guinea pig serum insensitivity). Further modifications

TABLE VII

The Failure of Various Amino Acids Added to Eagle's Medium to Prevent the Development of Guinea Pig Insensitivity in 6C3HED Cells

Amino acid	Concentration	Incubation period preceding demonstration of guinea pig serum insensitivity*	Amino acid	Concentration	Incubation period preceding demonstration of guinea pig serum insensitivity
	mg/liter	*wks.*		*mg/liter*	*wks.*
L-Alanine	200	3, 4	L-Lysine	200	3, 4
L-Arginine	200	3, 4	L-Methionine	200	3
D-Asparagine	200	5	L-Ornithine	50	5
L-Aspartic acid	20 to 200	See text	L-Phenylalanine	200	3, 4
L-Citrulline	50	5	L-Proline	200	3, 4
Glycine	200	3	L-Serine	200	5
L-Histidine	200	3, 4	L-Threonine	50	5
L-Leucine	200	3, 4	L-Tyrosine	200	3, 4

* The incubation periods shown are those for the first demonstration of guinea pig serum insensitivity in cells of each culture. In all cases controls using cells grown in unmodified Eagle's medium showed guinea pig serum insensitivity after incubation periods no shorter than in the media supplemented with the amino acids under test. Where 2 figures are shown each represents the result of a separate experiment.

of this medium contained 20.0, 50.0, and 100.0 mg of L-aspartic acid/liter, together with equimolecular amounts of ammonium ion (as ammonium citrate). Medium for the control culture contained no added L-aspartic acid, and in it 6C3HED cells behaved in the usual way, at first entering a latent phase; after 3 weeks, when active proliferation had begun, cells from this culture were shown to be completely insensitive to guinea pig serum. The cultures containing L-aspartic acid grew more vigorously than the control. After 3 weeks the cells cultured with 20.0 mg of L-aspartic acid/liter showed only partial insensitivity to guinea pig serum and cells in cultures containing 50.0 and 100.0 mg/liter were still completely sensitive. But, on retesting after 7 weeks' incubation, the cells from all cultures had become insensitive to guinea pig serum.

Since L-glutamine has been shown to contribute its amide group to the syn-

thesis of purines in cells grown in tissue culture (7), the possibility was tested that in 6C3HED cells the amide group of L-asparagine might have a similar synthetic function. A number of purines and their nucleotides, and purine precursors, were incorporated in the culture medium. As will be seen from the results shown in Table VIII, none of the added substances had any effect in preventing the development of guinea pig serum insensitivity by the cultured cells, although a few substances, notably 4-amino-5-imidazolecarboxamide hydrochloride and ammonium citrate, appeared to produce some degree of

TABLE VIII

Tests for the Ability of Various Substances to Prevent Development of Guinea Pig Serum Insensitivity by 6C3HED Cells in Tissue Culture in Eagle's Medium

Insensitivity developed in the presence of 0.35 mM concentrations of the following substances when cultured cells were tested after 5 weeks' incubation

Purine precursors	Purines	Pyrimidine precursor	Pyrimidines	Purine and pyrimidine mixture
Ammonium citrate	Adenine	Orotic acid	Cytosine	Orotic acid and adenine
4-Amino-5-imidazole-carboxamide HCl*	Adenosine 5′-monophosphate		Cytidine 5′-monophosphate	
Hypoxanthine	Guanine		Thymine	
Inosinic acid	Guanosine 5′-monophosphate		Thymidine 5′-monophosphate	
	Xanthine		Uracil	
			Uridine 5′-monophosphate	

The control 6C3HED cells in Eagle's medium with no added substance were insensitive to guinea pig serum when tested after 5 weeks' incubation.

* Cultured cells tested after 4 weeks' incubation.

growth stimulus. Similarly a number of pyrimidine bases and nucleotides, and their precursors failed to influence the development of guinea pig serum insensitivity.

In other experiments amino sugars were tested as L-asparagine substitutes. D-Glucosamine and chondroitin sulfate both at concentrations of 100.0 mg/liter failed to influence the growth characteristics or the development of guinea pig serum insensitivity in cells cultured in media to which they had been added. D-Galactosamine at a concentration of 100.0 mg/liter proved toxic to the cultured cells.

Conclusions from the entirely negative results of these experiments must be treated with reserve, especially as it was not shown that the cell membranes of

the 6C3HED cells were permeable to the various substances examined as substitutes for L-Asparagine. However, the results provide no suggestion that the major role of L-asparagine in the metabolism of guinea pig serum sensitive cells is in the synthesis of any amino acid, purine, pyrimidine, or amino sugar which was tested.

Fischer found that growth *in vitro* of lymphoma L5178Y, free from layers of feeder cells, only occurred if a high concentration of folic or folinic acid was present (8). Eagle's medium contains 1.0 mg/liter folic acid. Concentrations ranging from this to 20.0 mg/liter provided no growth stimulus for 6C3HED cells and failed to prevent the development of guinea pig serum insensitivity. Folinic acid in media containing graded concentrations from 0.05 to 2.0 mg/liter was similarly devoid of effect.

Now, the experiments so far described showed that 6C3HED cells sensitive to guinea pig serum require L-asparagine for growth *in vitro*, and that no other substance tested was able to replace it. They suggested that if inhibition of cells of Lymphoma 6C3HED by guinea pig serum was due to its L-asparaginase activity, as indicated by the evidence in the previous paper, then the simultaneous administration of L-asparagine *in vivo* might be able to neutralize the antilymphoma effect.

The Effect of the Administration of L-Asparagine on the Antilymphoma Activity of Guinea Pig Serum.—The possibility that L-asparagine might be able to reverse the antilymphoma effect of guinea pig serum was studied in detail in 7 separate experiments using different treatment techniques. In some experiments a slight effect in reversing tumor inhibition was observed but other experiments failed to demonstrate any inhibition whatsoever. 2 representative experimental results will be quoted.

In the first experiment 10 groups of 4 ZBC mice were implanted with 50,000 6C3HED cells in the right and left flanks in the usual way. 2 groups were set aside as untreated controls, animals in 3 other groups were treated with guinea pig serum, being given intraperitoneal injections respectively of 1.0, 0.5, and 0.25 ml 4 hours later. Animals in 5 exactly comparable groups were given subcutaneous injections of 100.0 mg L-asparagine, 1 and 10 hours after the tumor cells had been implanted. The injections, each of 1 ml of supersaturated L-asparagine solution, were given into the loose subcutaneous tissue behind the shoulders. Tumors appeared in untreated controls and in animals receiving only L-asparagine on the 11th to 12th day. 1 ml of guinea pig serum suppressed all implanted tumors regardless of whether or not any L-asparagine had also been given. 4 out of 8 tumors implanted were completely suppressed by 0.5 ml of guinea pig serum given alone, in 1 mouse both implanted tumors were completely suppressed; and in others the appearance of tumors was delayed 6 to 7 days after the controls. When L-asparagine was given in addition to 0.5 ml of guinea pig serum only 2 of the 8 tumors implanted were completely suppressed,

the remainder being delayed for 4 to 10 days after the controls. In groups of mice given 0.25 ml of guinea pig serum alone, a delay of 1 to 3 days was observed in the appearance of tumors, compared with the controls, but no delay was observed in animals also given L-asparagine.

A second experiment was performed using groups of ZBC mice, each implanted intraperitoneally with 50,000 6C3HED cells. A single injection of 0.5 ml of guinea pig serum was given subcutaneously 6 hours after tumor cell implantation and 6 intraperitoneal injections of 0.25 ml of 0.3 M L-asparagine were given at 2 hourly intervals after this, followed by 3 similar injections at 6 hourly intervals. A final injection of L-asparagine was given 44 hours after tumor implantation. In 6 untreated controls, all had obvious ascites on the 13th day. Of 7 animals given only guinea pig serum 4 had obvious ascites, and of 6 given guinea pig serum and L-asparagine only 2 had any evidence of ascites. In other groups of mice given guinea pig serum in amounts of 1.0 and 2.0 ml, again L-asparagine failed to show any antagonism to the tumor inhibitory action of the guinea pig serum.

DISCUSSION

The experiments here described show that 6C3HED cells cultured in Eagle's medium initially required L-asparagine for growth. They also show that in the absence of L-asparagine the lymphoma cell population changed within a period of 2 to 6 weeks to become independent of an external source of the amino acid. Cells from these cultures were now found to be completely insusceptible to the effects of guinea pig serum *in vivo*, and remained so even after repeated animal transfers.

The principal purpose of the present paper is to relate the development of L-asparagine independence to the loss of the character of guinea pig serum sensitivity. That the same underlying alteration or alterations within the cell may be responsible in both cases is shown by the following findings. First, as nearly as the experimental methods can determine, the ability of 6C3HED cells to proliferate in tissue culture in the absence of L-asparagine begins at the same time as they have been demonstrated to lose sensitivity to guinea pig serum. Secondly, 6C3HED cells remain sensitive to guinea pig serum when grown in tissue culture in the presence of relatively low concentrations of L-asparagine, such as occur physiologically in the plasma of several species which have been examined (9, 10). Furthermore, L-asparagine was highly specific in preventing the development of guinea pig serum insensitivity in the cultured cells and no other substance tested had any similar effect. Thirdly, cells of a subline of lymphoma 6C3HED, made insensitive to guinea pig serum *in vivo* by treatment with sublethal quantities, were able to proliferate vigorously in culture media containing no added L-asparagine, and without any period of latency such as that occurring with cells of the original guinea pig serum sensitive line.

There is no decisive evidence of whether the development of guinea pig serum insensitivity or L-asparagine independence occurs entirely by selection of variants in the original cell population or by the direct induction of an altered property in the 6C3HED cells by the conditions of culture. There is undoubtedly some degree of selection amongst the cells placed in culture, for on examination their number fell within 2 weeks to as few as $\frac{1}{300}$ of that originally inoculated. But these survivors did not immediately show proliferation, instead a further latent period of 30 days elapsed in which the number of cells in the culture remained virtually unchanged, before the very rapid increase in cell numbers occurred. The character of guinea pig serum insensitivity developed in culture was certainly not the result of a temporary adaptation or enzyme induction, for it was maintained through 14 transfer generations *in vivo* and was not reversed by growth in media containing optimal concentrations of L-asparagine.

L-Asparagine is considered a "non-essential amino acid" in animal diets, and *in vitro*, cell lines derived from non-neoplastic tissue have not been shown to require this amino acid for growth. Yet, by contrast, cells of lymphoma 6C3HED newly placed in culture and cells of certain other tumors too, fail to proliferate in tissue culture unless an external source of L-asparagine is provided. Neuman and McCoy (11) found that in addition to other amino acids, L-asparagine was necessary *in vitro* for the growth of the Walker carcinosarcoma 256, significantly, in view of the present findings, a guinea pig serum sensitive cell line (12). McCoy and coworkers later showed that 2 other tumors, the Jensen sarcoma and a rat hepatoma induced by methyldimethylaminoazo benzene, included L-asparagine amongst their growth requirements in culture (13, 14). The Novikoff hepatoma, however, had no such requirement (15). Recently Haley, Fischer, and Welch (16) identified L-asparagine as a growth-promoting substance in the protein hydrolysate which was a constituent of the medium used for culturing cells of the mouse lymphoma L5178Y.

But although in the present experiments 6C3HED cells initially required L-asparagine for growth *in vitro*, after a latent period cultures consistently became able to grow in its absence. Puck and Fisher (17), Haff and Swim (18) and Chang (19, 20) have described a number of examples of the appearance of variant cells in tissue culture able to grow in the absence of previously essential metabolites. Few studies have been made on the adaptation of cell cultures to growth in the absence of normally required amino acids, however of particular interest in relation to the experiments described in the present paper, are findings of McCoy *et al.* (21) using the Jensen sarcoma. Cells from this tumor had been found, as stated earlier, to require L-asparagine for growth *in vitro*, but on preparing cultures in media to which no L-asparagine had been added 2 sublines of variant cells were obtained which were able to proliferate in the deficient medium. As in the present experiments these variants appeared only after a prolonged period of latency.

Evidence presented in the companion paper indicates that the L-asparagine activity of guinea pig serum is responsible for the inhibition of cells of lymphoma 6C3HED. This view is supported in the present experiments by showing that dependence on an external source of L-asparagine by 6C3HED cells in tissue culture is closely related to their sensitivity to guinea pig serum on subsequent testing *in vivo*. But if L-asparaginase brings about tumor inhibition, how does it do so? Does the enzyme lower the level of L-asparagine in the extracellular fluids of the host so that the tumor cells die from deprivation of this essential nutriment? The finding in a number of experiments that L-asparagine is unable to significantly reverse the tumor inhibitory effects of guinea pig serum argues against this possibility, though it may be that with a high rate of clearance of L-asparagine in the treated animals the amounts given were insufficient to provide an adequate cover during the whole period in which the L-asparaginase activity was present. But it is possible that if L-asparaginase is the tumor inhibitory agent in guinea pig serum, then its action is not simply in lowering the level of L-asparagine in the tissue fluids surrounding the 6C3HED cells, but to act either at the surface or inside the tumor cells to interfere in some essential cell process in which L-asparagine is involved.

SUMMARY

Cells of the original line of lymphoma 6C3HED, which regularly prove susceptible to the effects of guinea pig serum *in vivo*, were cultured in Eagle's medium devoid of L-asparagine; after a latent period of 2 or more weeks, during which time the cell population declined markedly, some of the cells began to proliferate, and thereafter continued vigorous growth. On implantation into mice the proliferating cells were found, however, to have completely and permanently lost their susceptibility to the effects of guinea pig serum.

By contrast, when cultures of the original line of 6C3HED cells were prepared in Eagle's medium to which L-asparagine was added in a concentration of 20.0 mg/liter or more, they proliferated vigorously from the beginning; after long periods of growth in the enriched medium *in vitro* they remained susceptible to the effects of guinea pig serum upon test *in vivo*. Other amino acids, purines, and pyrimidines were unable to substitute for L-asparagine in this relation. Furthermore, a variant subline of 6C3HED cells which had become insensitive to guinea pig serum under *in vivo* conditions did not require L-asparagine for growth in tissue culture.

It seems plain from the findings as a whole, that in 6C3HED cells, L-asparagine dependence *in vitro* is associated with the *in vivo* character of guinea pig serum sensitivity, and conversely L-asparagine independent variants are insusceptible to the effects of guinea pig serum. The implications of the findings complement those of a companion paper in which direct evidence is provided that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects.

I am grateful to Dr. John G. Kidd, in whose laboratory this work was performed, for his advice and generous encouragement.

Mrs. P. Moy, Mrs. D. Yvars, and Mr. J. Sklarsh provided valuable technical assistance.

BIBLIOGRAPHY

1. Kidd, J. G., Effects of arsenic-azoproteins on mouse lymphoma cells *in vivo*. With observations on the effects of other "anti-lymphoma" agents, and on the susceptibility to these effects of lymphoma cells of various types, *J. Exp. Med.*, 1958, **108,** 665.
2. Schreck, R., A method for counting the viable cells in normal and in malignant cell suspensions, *Am. J. Cancer*, 1936, **28,** 389.
3. Paul, J., Cell and Tissue Culture, E. & S. Livingstone, Edinburgh, 1959.
4. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A. E., Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture, *J. Biol. Chem.*, 1957, **226,** 191.
5. Guerin, L. F., and Kitchen, S. K., Adaptation of 6C3HED tumor cells to culture *in vitro*, *Cancer Research*, 1960, **20,** 344.
6. Trevelyan, W. E., and Harrison, J. S., Studies on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates, *Biochem. J.*, 1952, **50,** 298.
7. Salzman, N. P., Eagle, H., and Sebring, E. D., The utilization of glutamine, glutamic acid, and ammonia for the biosynthesis of nucleic acid bases in mammalian cell cultures, *J. Biol. Chem.*, 1958, **230,** 1001.
8. Fischer, G. A., Studies of the culture of leukemic cells *in vitro*, *Ann. New York Acad. Sc.*, 1958, **76,** 673.
9. Barry, J. M., Asparagine in blood plasma, *Nature*, 1953, **171,** 1123.
10. Tallan, H. H., Moore, S., and Stein, W. H., Studies on the free amino acids and related compounds in the tissues of the cat, *J. Biol. Chem.*, 1954, **211,** 927.
11. Neuman, R. E., and McCoy, T. A., Dual requirement of Walker carcinosarcoma 256 *in vitro* for asparagine and glutamine, *Science*, 1956, **124,** 124.
12. Kwak, K. S., Jameson, E., Ryan, R. M., and Kurtz, H. M., The effect of varying implant cell numbers on the inhibitory activity of guinea pig serum on Walker carcinosarcoma 256 in the rat, *Cancer Research*, 1961, **21,** 44.
13. McCoy, T. A., Maxwell, M., and Kruse, P. F., The amino acid requirements of the Jensen sarcoma *in vitro*, *Cancer Research*, 1959, **19,** 591.
14. McCoy, T. A., and Maxwell, M., Some nutritional aspects of a 3′-methyl-4-dimethylaminoazobenzene–induced hepatoma in vitro, *J. Nat. Cancer Inst.*, 1959, **23,** 385.
15. McCoy, T. A., Maxwell, M., and Kruse, P. F., Amino acid requirements of the Novikoff hepatoma *in vitro*, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 115.
16. Haley, E. E., Fischer, G. A., and Welch, A. D., The requirements for L-asparagine of mouse leukemia cells L5178 in culture, *Cancer Research*, 1961, **21,** 532.
17. Puck, T. T., and Fisher, H. W., Genetics of somatic mammalian cells. I. Demonstration of the existence of mutants with different growth requirements in a human cancer cell strain (HeLa), *J. Exp. Med.*, 1956, **104,** 427.
18. Haff, R. F., and Swim, H. E., Isolation of a nutritional variant from a culture of rabbit fibroblasts, *Science*, 1957, **125,** 1294.

19. Chang, R. S., Isolation of nutritional variants from conjunctival and He La cells, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 818.
20. Chang, R. S., Differences in inositol requirements of several strains of He La, conjunctival and amnion cells, *Proc. Soc. Exp. Biol. and Med.*, 1958, **99,** 99.
21. McCoy, T. A., Maxwell, M., Irvine, E., and Sartorelli, A. C., Two nutritional variants of cultured Jensen sarcoma cells, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 862.