ALTERATIONS IN PROTEIN AND NUCLEIC ACID METABOLISM OF LYMPHOMA 6C3HED-OG CELLS IN MICE GIVEN GUINEA PIG SERUM*

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PLATES 8 TO 10

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Serum from normal guinea pigs regularly brings about the necrosis of Lymphoma 6C3HED cells of Gardner's original line when it is injected intraperitoneally in C3H mice in which the lymphoma cells are proliferating in the subcutaneous tissues, though it has no such effect when held in contact with the lymphoma cells during periods of several hours in vitro (1, 2). Much evidence has recently been found to indicate that the enzyme asparaginase, long known to be present in large amounts in the serum of guinea pigs but absent from the serum of mammals of other species (3), is responsible for its antilymphoma effects (4, 5), probably bringing these about in vivo by converting the available asparagine of the host to aspartic acid that cannot be assimilated by the lymphoma cells (6). The latter, dependent upon asparagine for protein synthesis (7) and for survival and growth in vitro (5), then die, their death resulting presumably from asparagine deprivation induced by the injection of guinea pig serum in vivo. In the work now to be reported it has been found that heated guinea pig serum, injected intraperitoneally in mice in which Lymphoma 6C3HED-OG cells (of Gardner's original line) are growing in the peritoneal cavity, promptly induces a striking alteration in the protein metabolism of the proliferating lymphoma cells, as disclosed by studies with radioactive valine; also that alterations in nucleic acid metabolism become manifest in the asparagine-dependent -OG cells after longer exposures to the effects of heated guinea pig serum in vivo, perhaps in consequence of a primary inhibition of protein synthesis. Because of the unique character and notable power of the effects of guinea pig serum (asparaginase) on cancers of various sorts (1–23), the findings have more than specialized interest.

Methods and Materials

General Plan of the Experiments.—Lymphoma 6C3HED cells of Gardner's original line (hereinafter Lymphoma 6C3HED-OG cells), which are known to be susceptible to the effects

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of guinea pig serum in vivo (1, 2), and to require asparagine for growth and for protein synthesis in vitro (5, 7), were implanted intraperitoneally in young adult C3H/Jax mice of the Heston line, approximately one million cells per mouse. For control purposes in certain experiments a comparable number of Lymphoma 6C3HED-RG1 cells, which are not susceptible to the effects of guinea pig serum in vivo (7, 24), and do not require asparagine for growth in vitro (5), were likewise implanted intraperitoneally in C3H/Jax mice. 5 or 6 days later, when the lymphoma cells had proliferated to the extent that some 75 to 150 million of them were present in the peritoneal cavity of each implanted host, 1 ml of pooled normal guinea pig serum that had been heated at 56°C for 30 min was injected intraperitoneally in each experimental mouse and 1 ml of normal horse serum that had also been heated at 56°C for 30 min was injected intraperitoneally in some of the control mice, others remaining untreated. In these relations it is known that pooled serum from normal guinea pigs regularly brings about the necrosis of Lymphoma 6C3HED-OG cells in vivo (1, 2); also that it contains asparaginase in large amount (3, 4), and that neither the antilymphoma principle of guinea pig serum, nor its asparaginase activity, is notably inactivated by heating at 56°C for 30 min (1, 4), though heating to this extent inactivates a complementlike factor in unheated guinea pig serum that sometimes has a deleterious effect upon Lymphoma 6C3HED cells in vivo (6). Normal horse serum is known to be devoid of effect on Lymphoma 6C3HED-OG cells in vivo (1) and also to be devoid of asparaginase activity (3, 4).

In studies on the fate of Lymphoma 6C3HED-OG cells in serum-treated mice, the hosts were killed and the lymphoma cells washed from the peritoneal cavities of the respective experimental and control animals and suspended in Eagle's medium to which horse serum (7 %) and asparagine (50 mg/liter) had been added. Counts were then made with the aid of a Coulter electronic counter to determine the yield of lymphoma cells from each mouse; further, the proportion of cells stained with eosin in small samples of each specimen was determined microscopically with the aid of an ordinary hemocytometer, this to provide indication of the proportion of nonviable cells (25). The remaining cells of each specimen were next centrifuged for 8 min at 900 RPM in the horizontal centrifuge; the pellets of centrifuged cells were fixed in osmium tetroxide pH 7.4, and embedded in Epon; sections 1 μ in thickness were then cut with the aid of the LKB ultra microtome and stained with toluidine blue for microscopic and photographic purposes.

In the metabolic studies, the incorporation of L-valine-C^{14} was used as a measure of protein synthesis (26), and the incorporation of tritiated thymidine was used to indicate deoxyribonucleic acid (DNA) synthesis (27–29); the synthesis of ribonucleic acid (RNA) was measured by the incorporation of uridine-5–H^3, since this was recently shown to be a specific precursor of RNA because the 5 position is labeled (30). One or another of the isotopes was injected intraperitoneally in measured amount in each of the respective experimental and control mice, whose peritoneal cavities already contained lymphoma cells that in most instances had been in contact with heated guinea pig serum or heated horse serum for various periods of time; after a labeling period of 5 or 30 min the hosts were killed and the lymphoma cells washed from their peritoneal cavities. The incorporation of the respective isotopes in the lymphoma cells was then determined by means of scintillation counting; autoradiography was used in addition in the studies on DNA metabolism.

Liquid Scintillation Counting.—As indicated above, one or another of the following isotopes, dissolved in 0.5 ml sterile isotonic saline solution in the indicated amounts, was injected intraperitoneally into the experimental and control mice: 4 or 8 μc of uniformly labeled L-valine-C^{14} 160 mc/mM (Nuclear-Chicago Corporation, Des Plaines, Illinois); 10 μc thymidine-H^3 3 c/mM (Schwarz Bioresearch Inc., Orangeburg, New York); 10 μc uridine-5–H^3, 5 c/mM (Nuclear-Chicago Corp.). After a labeling period of 5 or 30 min in vivo, the lymphoma cells were harvested, washed twice by centrifugation in Eagle's basal medium containing the

one of the following nonradioactive materials corresponding to the isotope employed: valine (141 μg/ml), thymidine (10 μg/ml), or uridine (10 μg/ml). Cell concentration was adjusted to approximately 1 million per ml with the aid of the Coulter electronic cell counter. Quadruplicate specimens each containing approximately two million cells in 2 ml were next set up; the cells were promptly precipitated with 2 ml cold 20% trichloroacetic acid (TCA), washed successively with 10 and 100% ethanol, and dissolved in 2 ml hydroxide of Hyamine.[1] The four dissolved specimens from each mouse were then added separately to 13 ml lots of a PPO-POPOP[2]–toluene mixture and these were placed in a Packard Tri-Carb liquid scintillation spectrometer for counting. Background was about 30 CPM for C^{14} and 50 CPM for H^{3}. Counting efficiency was 46 to 47% for C^{14} and about 7% for H^{3}.

Autoradiography.—On the 5th or 6th day following implantation, the mice were given 1 ml guinea pig serum (heated at 56°C for 30 min) intraperitoneally. Next, after a timed interval, intraperitoneal injections were made in the experimental and control mice of 0.5 ml sterile isotonic saline containing 12.5 μc thymidine-H^{3} 3 c/mM (Schwarz). After a labeling period of 30 min the mice were sacrificed, and the lymphoma cells were withdrawn from the peritoneal cavities; the cells were washed by centrifugation three times in Eagle's basal medium containing horse serum (2%) and nonradioactive thymidine (10 μg/ml). Smears of the washed cells were rapidly dried in cool air and fixed in 100% methanol for 5 min on glass slides covered with gelatin and chrome alum. The preparations were then coated with Ilford K-5 liquid emulsion (1 part emulsion plus 3 parts water). Following 7 to 10 days' exposure at −17°C the slides were developed in Kodak D-19 and stained with hematoxylin. Percentages of labeled cells were derived from counts of 1000 cells on each of duplicate slides.

RESULTS

The Fate of Lymphoma 6C3HED-OG Cells Exposed to the Effects of Guinea Pig Serum in Vivo.—From observations made previously it was known that substantial numbers of Lymphoma 6C3HED-OG cells remain viable following exposure of large populations of the lymphoma cells to guinea pig serum during 2 to 6 hr in vitro; also that the -OG cells of subcutaneous lymphomas of mice given large amounts of guinea pig serum intraperitoneally begin to die in situ within 8 hr (2). And further, that heated guinea pig serum injected intraperitoneally prevents the incorporation of L-asparagine-C^{14} by Lymphoma 6C3HED-OG cells growing intraperitoneally in favorable C3H hosts, much evidence indicating that the guinea pig serum acts in vivo by converting the host's L-asparagine to L-aspartic acid, which is not taken up by the lymphoma cells (6, 7). Hence the death in vivo of Lymphoma 6C3HED-OG cells that are known to be dependent upon L-asparagine for survival and growth in vitro (4) can presumably be looked upon as an ultimate manifestation of asparagine starvation.

In several experiments of the present work, observations were made that bear upon the fate of Lymphoma 6C3HED-OG cells growing intraperitoneally in favorable mouse hosts following the injection of heated guinea pig serum

[1] Hyamine, [p-(diisobutylcresoxethoxethyl)-dimethylbenzylammonium chloride], Rohm and Haas.

[2] PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2(5-phenyloxazolyl)-benzene, Nuclear-Chicago Corp.

intraperitoneally. Table 1, for example, shows the yield of lymphoma cells and the proportion of eosin-stained cells, an index of necrotic but morphologically-recognizable cells (25), in peritoneal washings from a number of mice of several experiments in which Lymphoma 6C3HED-OG cells had been exposed to the effects of guinea pig serum or horse serum in vivo during periods from 1 to 24 hr.

It can be seen from Table I that mice given horse serum 4 or 24 hr before they were sacrificed provided lymphoma cells quite comparable in number to those obtained from control untreated mice. So too mice given guinea pig serum 1, 2, 4, and 6 hr before they were sacrificed provided yields of lymphoma cells quite comparable to those procured from untreated control mice or from mice given horse serum. By contrast the mice given heated guinea pig serum 12, 16, or 24 hr before they were sacrificed provided comparatively low yields of lymphoma cells, these ranging from 1.20 to 27.53 millions, whereas yields ranging between 62.80 and 154.60 millions of cells were obtained from the untreated control mice and from those given horse serum.

The counts of eosin-stained cells also yielded information of value. It can be seen that the percentage of eosin-stained cells was 2 or less amongst cells procured from the untreated control mice; and so too amongst cells obtained from mice given horse serum, and from those given guinea pig serum 1 and 2 hr before sacrifice. The 2 mice of Experiment 2 that were given guinea pig serum 4 hr prior to sacrifice had 5 and 7% eosin-stained cells, respectively, while the animals of this experiment given guinea pig serum 6 and 12 hr prior to sacrifice had percentages of eosin-stained cells ranging from 8 to 12; the 2 mice given guinea pig serum 16 hr prior to sacrifice had eosin-stained cell counts of 12 and 0% respectively, and the 2 mice given guinea pig serum 24 hr before sacrifice had much reduced yields of lymphoma cells, all the remaining cells being unstained with eosin. The findings indicate that exposure to guinea pig serum in vivo during periods of 1 and 2 hr brought about little or no necrosis of the Lymphoma 6C3HED-OG cells; also that exposures to guinea pig serum during periods of 4 to 6 hr did not notably reduce the number of countable cells while bringing about the necrosis of some 5 to 12% of them, as determined by the eosin counts. Exposures of 12 and 16 hr following a single injection of 1 ml heated guinea pig serum resulted in marked diminution in the number of countable cells, while the proportion of eosin-stained cells amonst the remainder was 0 to 12%, as already stated. A single injection of 1 ml heated guinea pig serum 24 hr before sacrifice (Experiment 2: mice 29 and 30) brought about a marked reduction in the number of countable cells, but all were unstained in the eosin counts, as if the effects of the single injection of guinea pig serum given 24 hr before sacrifice had in the end become exhausted. By contrast, in mice given 4 ml of heated guinea pig serum in two injections of 2 ml each 24 and 12 hr prior to sacrifice and harvesting (animals 35 and 36 of Experiment 3,

TABLE I

Yields of Lymphoma 6C3HED-OG Cells From Peritoneal Cavities of Implanted C3H Mice Given Horse Serum or Guinea Pig Serum Intraperitoneally

Experiment No.	Mouse No.	Serum given i.p.	Interval between serum injections and sacrifice	Day sacrificed	Yield	Eosin-stained cells
			hr		*cells* $\times 10^6$	%
1	1	—	—	5	62.80	nd
	2	—	—	5	85.47	"
	3	—	—	5	87.18	"
	4	NHS	4	5	83.72	"
	5	"	24	6	139.60	"
	6	GPS	1	5	81.30	"
	7	"	2	5	72.77	"
	8	"	4	5	53.70	"
	9	"	24	6	1.20	"
2	10	—	—	5	84.10	0
	11	—	—	5	121.86	2
	12	—	—	5	76.40	2
	13	—	—	5	68.24	0
	14	NHS	4	5	140.72	1
	15	"	24	5	154.60	1
	16	GPS	1	5	79.44	2
	17	"	1	5	135.48	1
	18	"	1	5	171.90	0
	19	"	2	5	99.26	2
	20	"	2	5	111.10	1
	21	"	4	5	67.76	5
	22	"	4	5	95.92	7
	23	"	6	5	81.74	12
	24	"	6	5	90.28	8
	25	"	12	5	18.61	8
	26	"	12	5	19.91	10
	27	"	16	5	19.72	12
	28	"	16	5	7.66	0
	29	"	24	5	5.30	0
	30	"	24	5	11.30	0
3	31	—	—	5	143.98	0
	32	—	—	5	135.52	1
	33	NHS	24	6	100.04	0
	34	"	24	6	131.92	1
	35	GPS	24	6	7.87	10
	36	"	24	6	27.53	50
4	37	"	24	6	12.49	61
	38	"	24	6	15.04	45

All mice were implanted intraperitoneally with approximately 1.0 million Lymphoma 6C3HED-OG cells suspended in 1 ml of Eagle's medium to which horse serum (7 %) and asparagine (50 mg/liter) had been added. 4 or 5 days later the serums were injected as indicated and after a further interval the mice were sacrified and the lymphoma cells removed from the peritoneal cavities.

The serums were heated at 56°C for 30 min before use, see text. 1 ml of heated serum was given intraperitoneally in each instance except in mice 33 to 38 of Experiments 3 and 4, which received 4 ml of the respective serums in two equal amounts 24 and 12 hr prior to sacrifice.

nd, not determined.

and 37 and 38 of Experiment 4) the number of countable cells was greatly reduced as compared with the control values, while the proportion of eosin-stained cells in the remainder ranged from 10 to 61%.

Considered together, the findings recorded in Table I clearly indicate that the bulk of Lymphoma 6C3HED-OG cells growing intraperitoneally in mice given guinea pig serum 1, 2, 4, and 6 hr prior to harvesting remained during these periods intact and countable in the electronic counter, and unstained in the eosin counts. By contrast, the bulk of the lymphoma cells exposed in vivo to the effects of guinea pig serum during 12, 16, and 24 hr were either lysed, shrunken, or fragmented, their remains in any event being too small to register in the electronic cell counter; furthermore, in those animals injected with 2 ml guinea pig serum 12 and 24 hr before sacrifice, substantial proportions of the 6C3HED-OG cells remaining at the end took up dye in the eosin counts, hence were presumably necrotic.

In the light of previous observations (6, 7), the findings given thus far are compatible with the assumption that asparagine deprivation was brought about promptly in the Lymphoma 6C3HED-OG cells of mice given heated guinea pig serum intraperitoneally. During the first 6 hr of asparagine deprivation, however, the bulk of the -OG cells remained intact and countable in the electronic counter, and only a relatively small proportion of them were nonviable as indicated by the eosin counts (5 to 12% after 4 to 6 hr of exposure to the effects of guinea pig serum, as Table I shows). After 12 to 24 hr of exposure, however, the bulk of the cells were no longer countable in the electronic cell counter, and the cells remaining within the peritoneal cavities of mice given repeated injections of guinea pig serum during 24 hr were often nonviable, as the eosin counts indicated (Table I); so too morphologic studies, to be described next, showed plainly that the bulk of the -OG cells were necrotic following exposure in vivo to the effects of guinea pig serum during periods of 16 and 24 hr. Direct tests for viability, not given here in detail, made plain, however, that considerable numbers of the lymphoma cells remaining after 24 hr of contact with guinea pig serum in vivo were viable and of the -OG type.

As just mentioned, the morphologic studies provided further information about the sequence of events following injection of guinea pig serum intraperitoneally in mice in which Lymphoma 6C3HED-OG cells were growing within the peritoneal cavity. Fig. 1, for example, shows a number of Lymphoma 6C3HED-OG cells procured from a mouse given heated horse serum 4 hr before the cells were harvested for study. It can be seen that virtually all the lymphoma cells had vesicular nuclei containing usually a single large nucleolus, and finely granular cytoplasm with sharply outlined margins, i.e., the cells were intact and essentially unaltered. Cells from untreated mice, prepared in the same way, appeared precisely as did those of Fig. 1 except that 1 or 2% of the cells were pyknotic. So too some 99% of cells exposed during 15 min to the

effects of guinea pig serum in vivo appeared unaltered in microscopic preparations, while cells exposed to guinea pig serum for 1 hr in vivo showed a relatively slight increase in the percentage of cells exhibiting degenerative alterations (5 to 6%); after 2 or 4 hr of contact with guinea pig serum in vivo, approximately 90% of the lymphoma cells were still unaltered in microscopic preparations (Figs. 2 and 3). By contrast, cells exposed to guinea pig serum during 16 or 24 hr in vivo were greatly altered, the majority being frankly necrotic and most of the others showing advanced cytoplasmic vacuolization. Fig. 4, for example, shows unaltered Lymphoma 6C3HED-OG cells procured from the peritoneal cavity of a mouse given 2 ml of heated horse serum 24 hr before sacrifice and 2 ml horse serum 12 hr before sacrifice, while Fig. 5 shows Lymphoma 6C3HED-OG cells procured from the peritoneal cavity of a mouse given 2 ml of heated guinea pig serum 24 hr prior to sacrifice, and 2 ml 12 hr before sacrifice. It can be seen that the bulk of the cells from the mouse intensively treated with heated guinea pig serum were in general greatly altered (Fig. 5), having either pyknotic nuclei or copiously vacuolated cytoplasm. Direct tests, however, not given here in detail, showed that populations of cells harvested from the peritoneal cavities of mice given 2 ml guinea pig serum 24 hr prior to sacrifice and 2 ml 12 hr prior to sacrifice contained substantial numbers of viable lymphoma cells of -OG type, as already stated.

Alterations in Protein Synthesis by Lymphoma 6C3HED-OG Cells Growing in the Peritoneal Cavities of Mice Given Guinea Pig Serum Intraperitoneally, as Determined by Incorporation of L-*Valine-*C^{14}.—In experiments previously done, Lymphoma 6C3HED-OG cells required extrinsic L-asparagine in order to carry out unaltered protein synthesis in vitro, while the cells of Lymphoma 6C3HED-RG1, resistant to the effects of GPS in vivo, and independent of need for asparagine for survival and growth in vitro, incorporated L-valine-C^{14} into protein in vitro to the same degree whether or not L-asparagine was added to the medium (7). Experiments were next done to learn to what extent Lymphoma 6C3HED-OG cells synthesize protein in vivo when deprived of asparagine through the injection of guinea pig serum; comparable tests were also made with 6C3HED-RG1 cells. The -OG and -RG1 cells growing intraperitoneally in C3H mice were first exposed to the effects of heated guinea pig serum given intraperitoneally for periods of 0, 15, 60, and 120 min before "pulse" labeling, the zero minute exposure being one in which isotope (8 μc, L-valine-C^{14}) was given intraperitoneally immediately following injection of the guinea pig serum. A 5 min labeling period was allowed, after which 1 ml of Eagle's basal medium containing 141 μg valine ml was injected intraperitoneally in each mouse to stop incorporation of the label through competition by the unlabeled amino acid.

Table II shows the results of two such experiments. It can be seen that the -OG cells labeled immediately following administration of the guinea pig serum

(zero minute specimens) incorporated L-valine-C^{14} to an extent equal to or greater than that manifested by -RG1 cells under comparable conditions. Following 15 min of exposure to guinea pig serum, however, the -OG cells

TABLE II

Incorporation of L-valine-C^{14} by Lymphoma 6C3HED-OG and -RG1 Cells in Vivo, as Determined by Liquid Scintillation Counting: Effects of Guinea Pig Serum

Exp. No.	Mouse No.	Cells implanted	Yield (day 5)	Duration of exposure to GPS in vivo	Incorporation of L-valine-C^{14} CPM	
					Average	Range
			cells $\times$ *10^6*	*min*		
1	1	-OG	81.26	0	4006	3808–4205
	2		84.30	15	2981	2924–3033
	3		92.78	60	2027	1991–2098
	4		114.90	120	1400	1316–1468
	5	-RG1	106.70	0	3685	3470–3882
	6		133.62	15	3066	3020–3160
	7		121.48	60	3279	3071–3443
	8		141.54	120	3477	3428–3563
2	9	-OG	93.76	0	5680	5385–5999
	10		90.64	15	2170	2107–2245
	11		69.68	60	2265	2158–2347
	12		61.88	120	1520	1472–1584
	13	-RG1	113.24	0	3209	3044–3339
	14		120.62	15	4806	4540–5180
	15		86.16	60	4442	4360–4490
	16		123.04	120	7675	7565–7749

C3H mice were implanted intraperitoneally with approximately 1 million lymphoma cells of the respective types 5 days prior to the experiments. On the day of the experiment the mice received 1 ml heated guinea pig serum (GPS) (56°C, 30 min) 0 to 120 min prior to receiving 8 μc and 5.86 μg L-valine-C^{14} (160 mc/mM) in 0.5 ml isotonic saline. 5 min later 1 ml Eagle's basal medium containing 141 μg valine/ml was given intraperitoneally in order to stop incorporation of the label through competition by the unlabeled amino acid; the mice were then sacrificed and the cells aspirated from the peritoneal cavities. The cells were then processed for liquid scintillation counting as described in the text.

CPM, counts per minute per 2 million cells, quadruplicate specimens.

incorporated much less of the valine-C^{14}, and much less than did the -RG1 cells in comparable tests. It can be seen that in Experiment 1 of Table II the -OG cells incorporated successively less of the valine-C^{14} after exposures of 15, 60, and 120 min to the effects of guinea pig serum, while in Experiment 2 the -OG cells seemed to incorporate slightly more of the isotope after 60 min of exposure than following exposure to guinea pig serum for only 15 min, though

incorporation by the cells exposed 120 min was again greatly diminished and comparable to the corresponding value of Experiment 1. In this connection it is noteworthy that in both experiments the -RG1 cells, after 15, 60, and 120 min of exposure to the effects of guinea pig serum in vivo, incorporated quite as much of the valine-C^{14} as did these cells in the zero minute specimens, though it can be seen that the CPM values varied considerably from animal to animal, owing doubtless to biological variations amongst the mice.

In several additional experiments -OG cells exposed to the effects of guinea pig serum in vivo during periods of 15 or 20 min regularly incorporated less than half as much L-valine-C^{14} as did such cells procured from untreated control mice or from mice given horse serum 15, 60, 120, and 240 min before sacrifice; further, -OG cells exposed to guinea pig serum in vivo during periods of 60 and 120 min regularly incorporated successively lesser amounts of the label, while after 240 min the incorporation was only about one-fifth the control values provided by -OG cells exposed to horse serum.

The findings as a whole leave little room for doubt that protein synthesis by Lymphoma 6C3HED-OG cells is markedly reduced after only 15 min of exposure to the effects of heated guinea pig serum in vivo, as indicated by their incorporation of L-valine-C^{14}; and that protein synthesis is reduced still further following asparagine deprivation induced with heated guinea pig serum in vivo during periods of 60 and 120 min, at times, that is to say, when the bulk of the -OG cells remained morphologically intact and viable. Under comparable conditions of exposure to guinea pig serum in vivo, the -RG1 cells, known to be resistant to the effects of guinea pig serum in vivo and independent of need for extrinsic asparagine for growth in vitro, incorporated L-valine-C^{14} abundantly, and hence can be said to have synthesized protein in undiminished amounts; in these relations previous studies have shown that the -RG1 cells incorporate valine-C^{14} abundantly in vitro in the absence of asparagine (7) though they fail to take up L-asparagine-C^{14} in vivo and in vitro in the presence of guinea pig serum, presumably owing to the conversion of the L-asparagine-C^{14} to L-aspartic acid-C^{14} that is not taken up by the lymphoma cells (6).

Alterations in DNA Synthesis by Lymphoma 6C3HED-OG Cells of Mice Given Guinea Pig Serum, as Determined by the Incorporation of Tritiated Thymidine.—In the Experiment of Table III the incorporation of thymidine-H^3 was determined in Lymphoma 6C3HED-OG cells that had been exposed in vivo to the effects of horse serum and guinea pig serum respectively during periods of 15, 60, 120, and 240 min; the cells of an untreated control mouse were also studied. It can be seen that in specimens of the cells procured from mice given horse serum 15, 60, 120, and 240 min prior to sacrifice, the thymidine was incorporated to the same degree as in cells procured from an untreated control mouse, the CPM values ranging from 10,580 to 20,310 in quadruplicate counts from each mouse given horse serum, compared with an average of 15,560 in the

specimens procured from the untreated mouse. The cells exposed in vivo to guinea pig serum during 15 and 60 min incorporated the tritiated thymidine in comparable amounts, the CPM values ranging from 8340 to 13,475; by contrast the -OG cells exposed 120 and 240 min to the guinea pig serum incorporated much less of the label, the counts at 120 min averaging 4059 and those at 240 minutes, 1933 CPM.

Liquid scintillation counting, as used in the experiment of Table III, provides data on populations of cells; it does not determine whether all the cells of a

TABLE III

Incorporation of Thymidine-H^3 by Lymphoma 6C3HED-OG Cells in Vivo, as Determined by Scintillation Counting: Effects of Guinea Pig Serum

Mouse No.	Yield of -OG cells	Heated serum i.p. 1 ml	Duration of exposure to serum in vivo	Incorporation of thymidine-H CPM	
				Average	Range
	$\times 10^6$		*min*		
1	69.44	—	—	15,560	15210–15880
2	169.22	Horse serum	15	17,240	16090–17890
3	94.56	" "	60	12,947	10575–14315
4	65.74	" "	120	17,425	14610–20310
5	90.48	" "	240	11,820	10580–13510
6	119.68	Guinea pig serum	15	12,390	10870–13475
7	41.78	" " "	60	10,189	8340–12210
8	63.48	" " "	120	4,059	3270–4460
9	53.72	" " "	240	1,933	1834–2056

Implantation and harvesting procedures identical with those of the experiments of Table II, q.v.

Labeling period 30 min.

Mice received 10 μc thymidine-H^3 in 0.5 ml saline.

CPM, counts per minute per 2 million cells, quadruplicate specimens for each mouse.

given specimen incorporate tritiated thymidine equally or whether there is noteworthy variation amongst the cells. To learn about this, autoradiography was done on -OG and -RG1 cells exposed to the effects of guinea pig serum in vivo; again the incorporation of thymidine-H^3 was used as a measure of DNA formation. The autoradiographs of -OG cells exposed to the effects of guinea pig serum revealed that the cells of the respective specimens could be easily separated into three categories. First were those so heavily labeled that they were virtually obscured by innumerable exposed silver grains; these were designated "heavily labeled" cells (Figs. 6, 7). A second category included cells covered by many fewer exposed silver grains, which were easily enumerated;

these were designated "lightly labeled" cells (Figs. 8, 9). The third class of cells was devoid of exposed silver grains; these were designated "unlabeled" cells.

Table IV shows that -OG cells exposed to guinea pig serum for 15 and 60 min were labeled like untreated control cells, or like -RG1 cells exposed to guinea pig serum, i.e. some 60% of the cells were heavily labeled, 5% or fewer were lightly labeled, and 33% or more were unlabeled, the latter being

TABLE IV

Incorporation of Thymidine-H^3 by Lymphoma 6C3HED-OG and -RG1 Cells in Vivo, as Determined by Autoradiography: Effects of Guinea Pig Serum

Mouse No.	Yield of cells	Duration of exposure to 1 ml heated GPS	Incorporation of H^3-thymidine		
			Heavily labeled cells*	Lightly labeled cells*	Unlabeled cells*
	$\times 10^6$		%	%	%
-OG 1	43.20	No serum	64	3	33
2	64.88	15 min	56	5	39
3	37.94	60 "	58	3	39
4	63.13	120 "	>1	60	40
5	44.30	240 "	>1	57‡	43
6	12.49	24 hr	1	>1	99
-RG1 1	121.46	No serum	56	6	38
2	82.50	15 min	60	5	35
3	110.74	60 "	60	2	38
4	169.90	120 "	53	3	44
5	157.62	240 "	51	4	45
6	nd	24 hr	55	10	35

Implantation procedures identical with those of the experiments of Table II, q.v. Harvesting procedures described in text.

Mice -OG 2 and 5, and -RG1 2 and 5 given 1 ml guinea pig serum (GPS); mice -OG 6 and -RG1 6 given 4 ml GPS in two equal doses 24 and 16 hr prior to labeling.

* See Figs. 6 to 9.

‡ Cells somewhat less heavily labeled than were those of the 120 min specimen.

obviously the proportion of the cell population normally not engaged in DNA synthesis during the "pulse" labeling period. After 120 min of exposure to guinea pig serum, however, there was a striking change in the labeling. The same percentage of cells was labeled as in the controls, (about 60%), but now virtually all of the labeled cells were lightly labeled rather than heavily labeled (Table IV, Fig. 8). The same was true of the -OG cells exposed to guinea pig serum for 4 hr (Fig. 9), though scrutiny of the preparations made plain that the cells of the 4-hr specimen were overlain by fewer exposed grains than were those of the 2-hr specimen. After 24 hr of exposure almost all the -OG cells were unlabeled. Of the cells surviving 24 hr of exposure, the 1% or less that

was heavily labeled may possibly have been nonneoplastic elements, though the possibility also deserves consideration that they may have been mutant or transformed cells of the -RG1 type.

Thus the evidence from scintillation counting and autoradiography indicates that -OG cells exposed to guinea pig serum in vivo did not show notable alterations in DNA synthesis during the early periods, i.e., after 15 and 60 min of exposure to the effects of guinea pig serum in vivo, when marked curtailment

TABLE V

Incorporation of Uridine-5-H^3 by Lymphoma 6C3HED-OG Cells in Vivo, as Determined by Scintillation Counting: Effects of Guinea Pig Serum

Mouse No.	Yield of -OG cells	Heated serum i.p. 1 ml (56°C, 30′)	Time in contact with serum in vivo	Incorporation of Uridine-5-H^3 CPM	
				Average	Range
	× 10^6		*min*		
1	123.82	—	—	5086	4945–5200
2	69.64	Horse serum	15	9597	9447–9770
3	93.50		60	7491	6760–8205
4	129.62	" "	120	4679	4640–4740
5	123.26	" "	120	5350	4790–5870
6	95.86	" "	240	4044	3920–4210
7	76.94	Guinea pig serum	15	6613	6245–6835
8	95.98	" " "	60	4970	4800–5150
9	119.96	" " "	120	4082	3962–4220
10	82.46	" " "	120	4308	4015–4535
11	119.02	" " "	240	2668	2560–2764

Labeling period 30 min.
Mice received 10 μc uridine-5-H^3 in 0.5 saline.
Other conditions like those in experiments of Table II.

of protein synthesis was already detectable. After 2 hr of exposure to the effects of guinea pig serum in vivo, however, although the proportion of cells taking up thymidine-H^3 remained unchanged, there was a marked decrease in the amount of thymidine taken up per cell, fewer than 1% of the cells being heavily labeled after 2 hr of exposure whereas 56 and 58% of the cells were heavily labeled following 15 and 60 min of exposure, respectively. After 4 hr of exposure the cells incorporated still less of the thymidine-H^3, the average CPM value of the 4-hr specimen being only half that of the 2-hr specimen and one-fifth that of the 15- and 60-min specimens (Table III), while some 60% of the cells were now lightly labeled and fewer than 1% were heavily labeled, as the autoradiographs showed (Table IV). Exposure to the effects of guinea pig serum in vivo

during periods of 2 and 4 hr manifestly did not prevent the initiation of DNA synthesis by a number of the lymphoma cells during these periods, for the same percentage of cells was labeled after such exposures as before. But the cells of a given proportion of the population (approximately 60%) exposed during periods of 2 and 4 hr were now lightly labeled rather than heavily labeled, as were the unexposed cells and those exposed during periods of 15 and 60 min, the findings indicating that the intensity of DNA synthesis per lymphoma cell was greatly reduced in the populations exposed during periods of 2 and 4 hr to the effects of guinea pig serum in vivo. After 24 hr of exposure, it can be seen (Table IV), that almost all the cells failed to take up the tritiated thymidine. The latter finding was not unexpected in light of the morphologic state of the cells after 24 hr of exposure (Fig. 5).

Alterations in RNA Synthesis by Lymphoma 6C3HED-OG Cells of Mice Given Guinea Pig Serum, as Determined by Incorporation of Uridine-5-H^3.—In an experiment comparable to those of the two preceding sections, the incorporation of uridine-5–H^3 by -OG cells was used to measure RNA formation. Table V shows the results. It can be seen that cells from the untreated control mouse and those from the mice given horse serum for various periods before sacrifice incorporated uridine-5–H^3 to the extent that the CPM values ranged from 3920 to 9770 with cells from the respective mice. So too the cells from mice given heated guinea pig serum for periods of 15, 60, and 120 min prior to sacrifice gave comparable CPM values; by contrast the cells exposed to guinea pig serum during 4 hr incorporated notably less of the isotope, the finding indicating that the ability of the -OG cells to synthesize RNA was reduced following asparagine deprivation induced by guinea pig serum in vivo during the 4 hr of exposure.

DISCUSSION

A number of observations now throw light on the means whereby guinea pig serum brings about the necrosis of certain lymphoma cells in vivo (1, 2). For example, it has long been known that guinea pig serum contains asparaginase in large amounts (3), and recent findings indicate that this is responsible for its antilymphoma effects (4, 5, 16, 17, 19, 21, 23). Further, certain lymphoma cells that are susceptible to the effects of guinea pig serum in vivo (1, 2), of which Lymphoma 6C3HED-OG cells provide an example, are known to be asparagine-dependent, i.e., to require adequate amounts of asparagine for protein synthesis and for growth in vitro (7, 5); other types of lymphoma cells, of which Lymphoma 6C3HED-RG1 cells are an example, are wholly resistant to the effects of guinea pig serum in vivo (7, 24), and do not depend upon asparagine for their ability to synthesize protein (7) or to grow in vitro (5). Considered together the findings provide basis for the supposition that the asparaginase of guinea pig serum, upon injection into mice in which lymphoma cells are proliferating, converts the available (extracellular) asparagine of the

host to aspartic acid that is not taken up by the lymphoma cells (6), thus bringing about an asparagine-free or asparagine-poor state in the milieu in which the lymphoma cells live.

Beginning some 8 to 24 hr following exposure to the effects of guinea pig serum in vivo, asparagine-dependent -OG cells regularly die in great numbers, as previous findings (2) and those here presented show; other evidence indicates that death of the lymphoma cells is presumably an ultimate manifestation of asparagine starvation (6). Long before necrosis occurs, however, striking changes take place in the protein metabolism of proliferating -OG cells when heated guinea pig serum is brought into contact with them in vivo. Indeed, in the experiments here reported, within 15 min following the injection of heated guinea pig serum into the peritoneal cavities of mice in which -OG cells were growing, protein synthesis by the lymphoma cells, as manifested by their ability to incorporate L-valine-C^{14}, was markedly curtailed, being only about half that of -OG cells exposed in vivo to the effects of horse serum under comparable conditions; further, following exposures to the effects of heated guinea pig serum in vivo for periods of 60, 120, and 240 min, the -OG cells incorporated successively less of the isotope.

DNA metabolism, by contrast, proceeded undiminished during 15 and 60 min following the injection of heated guinea pig serum, as was disclosed by the ability of some 60 % of the lymphoma cells, recovered at the respective times from mice given the guinea pig serum, and beforehand from an untreated control mouse, to take up large quantities of tritiated thymidine and become "heavily labeled" in autoradiographs. 2 and 4 hr after injection of the heated guinea pig serum, however, the amount of DNA synthesis per lymphoma cell was greatly reduced; for although 56 to 58% of the cells still incorporated enough tritiated thymidine to become lightly labeled in the autoradiographs, virtually all of them had by now lost their ability to incorporate enough thymidine to become heavily labeled. The findings, corroborated by liquid scintillation counts, suggest that DNA synthesis by growing Lymphoma 6C3HED-OG cells in vivo proceeds normally at a high level of intensity, leading to some 60% of the cells being heavily labeled at any given time; in the experiment of Table IV, the high level of intensity continued in lymphoma cells exposed for 15 and 60 min to the effects of heated guinea pig serum in vivo, but after 120 min of exposure the lymphoma cells synthesized DNA at a much reduced level, leading to the same proportion of cells being only lightly labeled in the autoradiographs, while the labeling was even less after 240 min of exposure.

Hence the observations of the present work have made plain that protein synthesis by 6C3HED-OG cells in vivo was promptly curtailed within 15 min following the injection of heated guinea pig serum; in comparable experiments, however, DNA synthesis proceeded undiminished for 60 min or more, and was curtailed only after 120 min of exposure to the guinea pig serum in vivo. The

findings suggest that the prompt reduction in protein synthesis may have been a primary consequence of asparagine deprivation in the lymphoma cells, which in turn led secondarily to the curtailment of DNA synthesis that became manifest later, as has been shown to occur in other systems (31–34). So too in further experiments: RNA synthesis by -OG cells proceeded unchanged during periods of 15, 60, and 120 min following the injection of heated guinea pig serum, but was notably diminished after 240 min, the findings again suggesting that the eventual curtailment of RNA synthesis may have occurred in response to prior reduction in protein or DNA synthesis.

SUMMARY

Lymphoma 6C3HED-OG cells, known from previous work to be susceptible to the effects of guinea pig serum in vivo and dependent upon extrinsic asparagine for protein synthesis and growth in vitro, remained for the most part morphologically intact and countable in the electronic cell counter following exposures of 1 and 2 hr to the effects of heated (56°C, 30 min) guinea pig serum injected into the peritoneal cavities of mice in which the lymphoma cells were growing rapidly; after exposures of 4 and 6 hr the bulk of the -OG cells remained still intact and countable in the cell counter, though by this time a small proportion of them (5 to 12%) proved stainable with eosin in wet preparations, hence were presumably nonviable.

After 12, 16, and 24 hr of exposure, however, the bulk of the -OG cells were either lysed or fragmented, to the extent that they did not register in the cell counter. Morphologic studies of the cells exposed 16 and 24 hr to the effects of heated guinea pig serum in vivo, disclosed that most of the cells then remaining were either frankly necrotic or greatly altered otherwise, marked vacuolation of the cytoplasm being the most conspicuous alteration in cells not yet obviously necrotic.

Long before the bulk of the Lymphoma 6C3HED-OG cells had become conspicuously changed morphologically following exposure to the effects of heated guinea pig serum in vivo, they manifested striking alterations in protein metabolism, as was disclosed by "pulse" studies with radioactive valine. For example, the protein metabolism of -OG cells, as measured by their incorporation of L-valine-C^{14}, was sharply curtailed following 15 min of exposure to heated guinea pig serum in vivo, as compared with valine incorporation by cells labeled immediately after exposure to the guinea pig serum. Following exposure to heated guinea pig serum during 60 min, -OG cells incorporated less than half as much L-valine-C^{14} as did cells labeled immediately after exposure, and the incorporation of L-valine-C^{14} was still less after 120 min of exposure. By contrast, Lymphoma -RG1 cells, known from previous work to be wholly insusceptible to the effects of guinea pig serum in vivo and independent of need for extrinsic asparagine for protein synthesis and growth in vitro, showed no curtail-

ment whatever of protein synthesis following exposures to the effects of heated guinea pig serum in vivo during periods of 15, 60, and 120 min. Reasons are given for considering the prompt inhibition of protein synthesis in the asparagine-dependent -OG cells a direct result of asparagine-deprivation induced in vivo by the injected guinea pig serum, the L-asparaginase of which presumably converted the available L-asparagine of the host to L-aspartic acid that was not taken up by the -OG cells.

The synthesis of deoxyribonucleic acid by Lymphoma 6C3HED-OG cells, as measured by the incorporation of thymidine-H^3, determined with the aid of liquid scintillation counting and autoradiography, was also altered by exposure of the lymphoma cells to the effects of heated guinea pig serum in vivo, though not during exposures of 15 and 60 min; only after an exposure of 120 min did the population of -OG cells incorporate notably less thymidine-H^3 than did control populations, though after 240 min of exposure the -OG cells incorporated less than one-fifth as much tritiated thymidine as had -OG cells exposed to heated guinea pig serum for 60 min or to heated horse serum for periods up to 240 min. Autoradiographs indicated that DNA synthesis by -OG cells normally proceeds at an intense level that leads to some 60% of these cells being heavily labeled in autoradiographs at any given time; after exposure to the effects of heated guinea pig serum during 2 and 4 hr in vivo, however, the lymphoma cells lost their ability to incorporate enough tritiated thymidine to become heavily labeled, but approximately the same proportion of them (56 to 58%) retained their ability to incorporate sufficient tritiated thymidine to become lightly labeled. The possibility is considered that the inhibition of DNA synthesis in the asparagine-dependent -OG cells exposed to the effects of heated guinea pig serum in vivo may be secondary to the previously manifest inhibition of protein synthesis.

Further, in tests of ribonucleic acid metabolism of Lymphoma 6C3HED-OG cells after exposure to the effects of heated guinea pig serum in vivo during periods of 15, 60, 120, and 240 min, the findings indicated that the ability of the lymphoma cells to synthesize RNA, as measured by their capacity to incorporate uridine-5–H^3, remained unaltered during the exposures of 15, 60, and 120 min, but was substantially reduced following 240 min of exposure.

The findings are considered in relation to the probability, disclosed in part by previous studies, that heated guinea pig serum brings about its effects upon Lymphoma 6C3HED-OG cells in vivo by providing active L-asparaginase in large amounts, which presumably converts the available (extracellular) asparagine of the host to aspartic acid, the latter not being taken up by the lymphoma cells in vivo or in vitro. Hence it seems likely that heated guinea pig serum in this way brings about a state of asparagine deprivation that is responsible for the sequential metabolic and morphologic alterations that become manifest in

asparagine-dependent Lymphoma 6C3HED-OG cells following their exposure to the effects of guinea pig serum in vivo, as here described.

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EXPLANATION OF PLATES

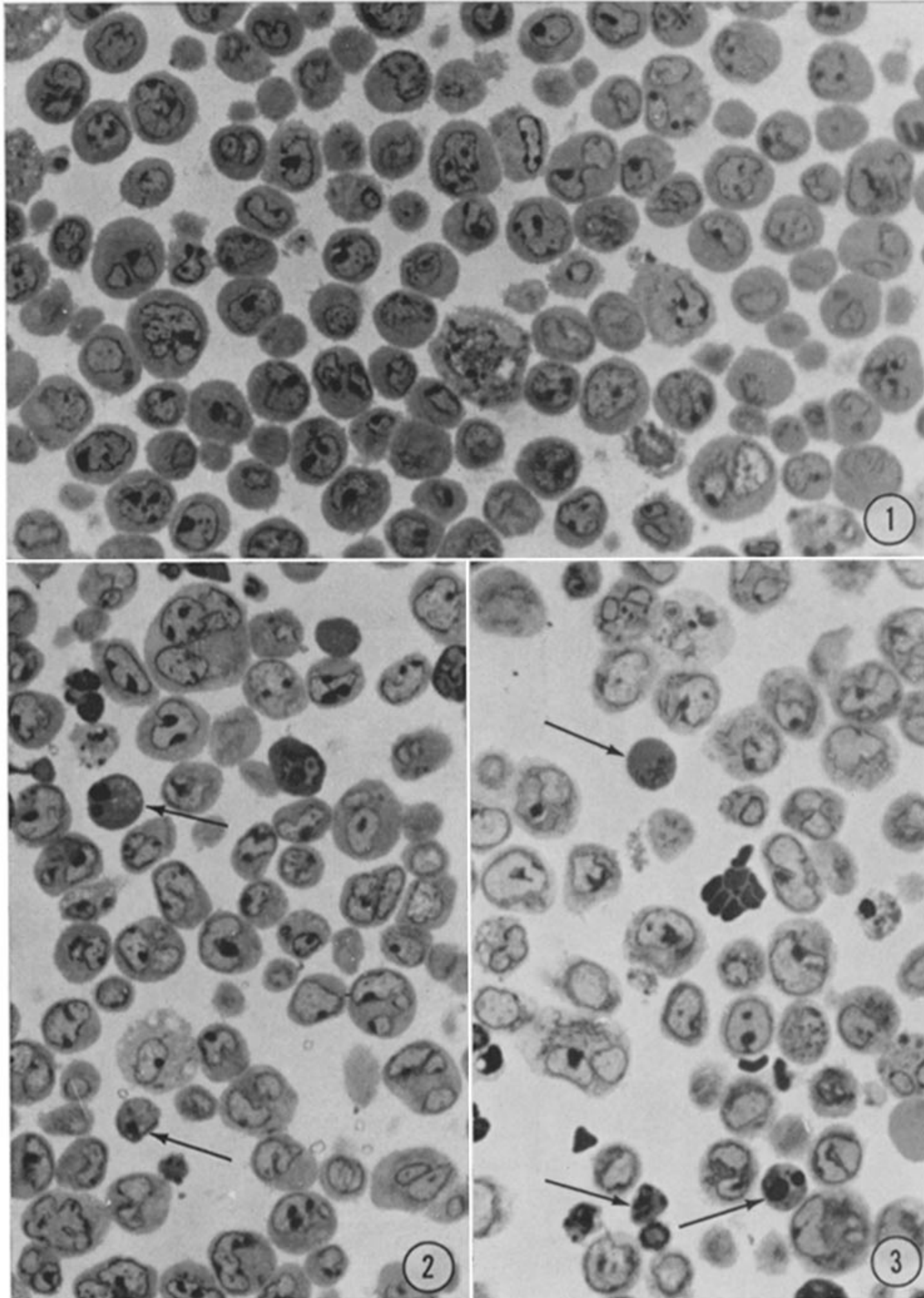
Plate 8

Figs. 1 to 3. Photomicrographs of pellets of Lymphoma 6C3HED-OG cells procured 5 days after implantation of 1 million cells intraperitoneally in C3H mice. Cell pellets fixed in OsO_4, embedded in Epon, cut at 1μ, and stained with toluidine blue. $\times$ 800.

Fig. 1. Cells exposed to heated (56°C, 30 min) horse serum for 4 hr in vivo. Generally homogeneous population. Large macrophage in center of field.

Fig. 2. Cells exposed to heated guinea pig serum for 2 hr in vivo. The majority of the cells are unaltered. At least two of the cells in the field are necrotic (arrows).

Fig. 3. Cells exposed to heated guinea pig serum for 4 hr in vivo. The majority of the cells are unaltered. Scattered necrotic cells (arrows). Clumped erythrocytes in center of field. Three individual erythrocytes scattered in lower portion of field.



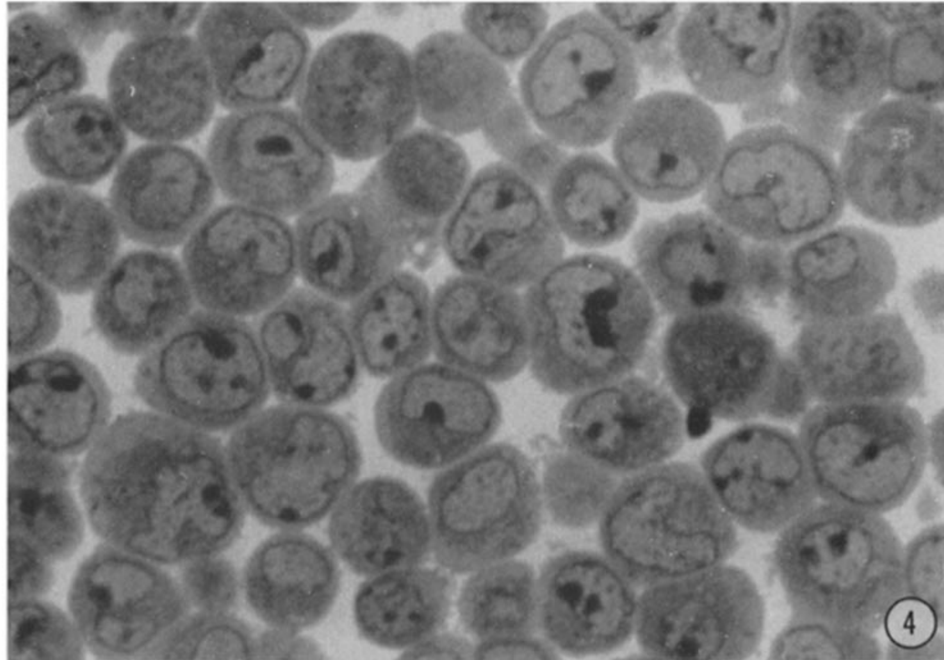
(Sobin and Kidd: Lymphoma 6C3HED-OG cells)

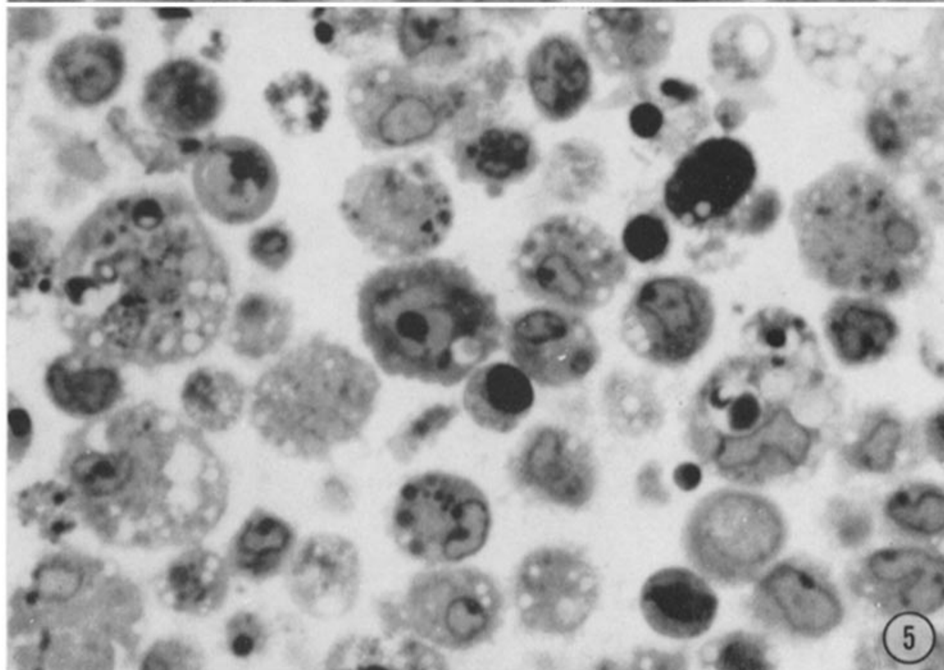
Plate 9

Figs. 4 and 5. Photomicrographs of pellets of Lymphoma 6C3HED-OG cells procured 6 days after implantation of 1 million cells. Fixed in OsO_4, embedded in Epon, cut at 1μ, and stained with toluidine blue. $\times$ 1260.

Fig. 4. Cells exposed to heated horse serum for 24 hr in vivo (2 ml, 24 hr and 2 ml, 12 hr prior to sacrifice). Cells devoid of signs of degeneration.

Fig. 5. Cells exposed to heated guinea pig serum for 24 hr in vivo (2 ml, 24 hr and 2 ml, 12 hr prior to sacrifice). Extensive necrosis and degeneration with nuclear pyknosis and cytoplasmic vacuolization most evident. Few cells are unaltered.





(Sobin and Kidd: Lymphoma 6C3HED-OG cells)

Plate 10

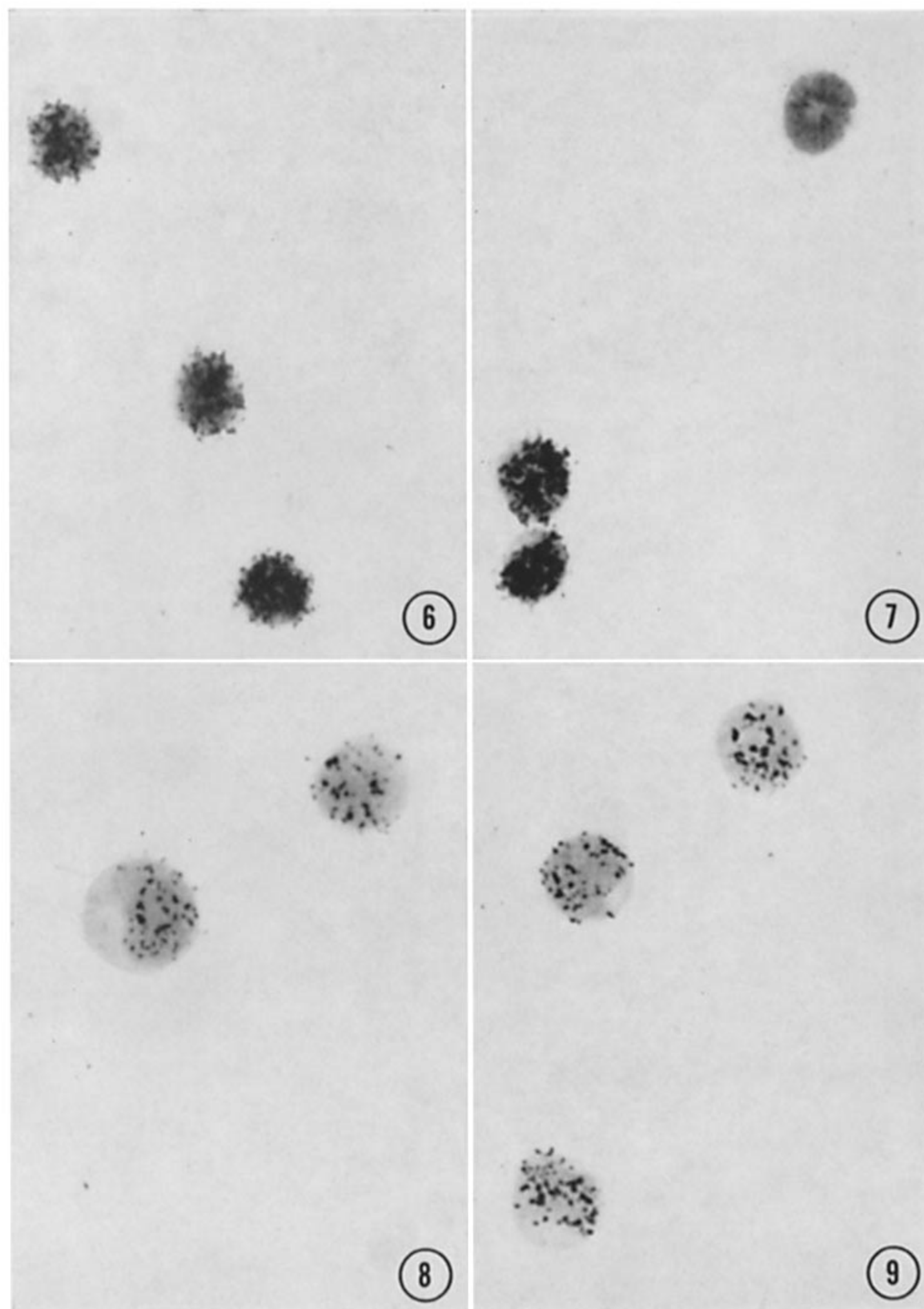
Figs. 6 to 9. Autoradiographs of smears of Lymphoma 6C3HED-OG cells after labeling in vivo with thymidine-H^3. Ilford K-5 emulsion. Exposure 1 wk. Hematoxylin. $\times$ 1000.

Fig. 6. Untreated lymphoma cells; heavily labeled.

Fig. 7. Cells exposed to heated guinea pig serum for 60 min. 2 heavily labeled cells and 1 unlabeled cell in mitosis.

Fig. 8. Cells exposed to heated guinea pig serum for 120 min. 2 lightly labeled cells.

Fig. 9. Cells exposed to heated guinea pig serum for 240 min. 3 lightly labeled cells.



(Sobin and Kidd: Lymphoma 6C3HED-OG cells)