REGRESSION OF TRANSPLANTED LYMPHOMAS INDUCED IN VIVO BY MEANS OF NORMAL GUINEA PIG SERUM

II. Studies on the Nature of the Active Serum Constituent: Histological Mechanism of the Regression: Tests for Effects of Guinea Pig Serum on Lymphoma Cells in Vitro: Discussion*

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The experimental findings of the associated paper have shown that guinea pig serum given intraperitoneally to mice with transplanted lymphomas of either of two types regularly brings about their regression. The studies now to be reported deal with the nature of the material in guinea pig serum which is responsible for the effect, and with the mechanism whereby this is brought about. The findings show that the active serum constituent is probably a protein, and that under certain experimental conditions its inhibitory effects are enhanced in the living animal by the concurrent injection of immune serum prepared by injecting mouse lymphosarcoma cells into rabbits, the observations together suggesting that the active constituent may be one or another of the components of complement. Further studies have shown that the lymphoma cells of tumors *in situ* promptly die and are resorbed following the injection of guinea pig serum intraperitoneally into hosts carrying them. Yet the lymphoma cells, when artificially suspended as individuals in physiological saline solution, regularly remained viable during long periods of contact *in vitro* with normal guinea pig serum. From these latter findings it seems certain that regression of the growths is brought about *in vivo* through some reaction in which the host and the active constituent of the guinea pig serum both participate. Further implications of the findings will be given in the discussion.

Methods and Materials

The 6C3HED lymphosarcoma, referred to in the associated paper, was employed exclusively in the experiments now to be reported. Salting out with

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ammonium sulfate, and other fractionation procedures, together with filter paper electrophoresis, micro-Kjeldahl analyses, and chymotrypsin- and heat-inactivation tests, were used, as will be described further on, in attempts to identify the constituent of guinea pig serum responsible for the regression. Microscopic studies were made of subcutaneous lymphosarcomas that were regressing following the intraperitoneal injection of guinea pig serum, and similar studies were also made of the viscera of the injected animals, together with those of suitable controls, to find out whether the injections had brought about any alterations in them. To learn whether the guinea pig serum acts directly upon the lymphoma cells, mixtures of the two were incubated *in vitro* for several hours, with subsequent implantation into susceptible hosts of saline suspensions of the cells that had been separated from the mixtures by gentle centrifugation.

For purposes of integration the charts and references of the two papers are number consecutively.

Characteristics of the Effective Constituent of Guinea Pig Serum

From the findings given in the associated paper it is plain that there are limitations to the usefulness of whole guinea pig serum as a means of inducing regression of experimental lymphomas *in vivo*. In the experiments of Charts 1 to 4 of Paper I, for example, it proved necessary to give repeated injections of relatively large quantities of the whole serum to mice in order to bring about regression of the growths, and even when these were given the regression was was not always permanent; furthermore, it proved impossible with the quantities of guinea pig serum available, to do more than stay temporarily the growth of Murphy-Sturm lymphosarcoma cells in rats (Chart 6). Moreover, although signs of reaction to the relatively large amounts of foreign protein given were not manifest in the animals of the present study, it seems probable that these might have been found had large numbers of the treated animals been studied more closely, and it seems almost certain, on the basis of much experience, that serious reactions would follow the injection of proportionate quantities of foreign serum into other mammalian species. Hence it seemed important to identify and concentrate the active constituent if possible, with a view to being able to inject effective amounts of it without injecting unnecessary foreign proteins at the same time. For these practical reasons, and for theoretical reasons also, a number of attempts were made to learn about the nature of the active material. Initially tests were made of the effects of heat on whole guinea pig serum containing it, while fractionation methods, to be described further on, were employed in attempts to isolate and concentrate the active material, and the effects of chymotrypsin on isolated fractions containing it were also studied.

The Effects of Heat.—Repeated observations made it plain that guinea pig serum that had been heated at 56°C. for 20 or 30 minutes regularly retained its power to induce regression of 6C3HED lymphosarcomas *in vivo.*

In the experiment of Chart 2, for example, 8 implanted mice of a group not shown in the chart were each given on days 4, 5, 6, 7, 8, 9, and 10, 0.5 cc. of guinea pig serum that had been heated at 56°C. for 30 minutes. On the day the injections were begun, the growths of these animals were quite comparable to those of the two charted groups; *i.e.*, 3 to 7 mm. across and moderately firm. Their subsequent course was precisely like that of the growths in animals given unheated serum (Group 2 of Chart 2)—that is to say, they promptly dwindled and disappeared, and all 8 hosts remained lively and devoid of palpable tumors throughout 120 days' observation.

Chart 9 shows the results of an experiment in which specimens of guinea pig serum that had been heated at 56°C. and 66°C. respectively, were tested, along with unheated specimens, for ability to alter the outcome of implantations with 6C3HED cells. It will be seen that the serum specimen that had been heated at 66°C. for 30 minutes had no effect on the growth of 6C3HED lymphosarcoma cells in 4C3H mice when 2.0 cc. of it was given to each of them 1 hour after the implantation of 2.5 million lymphosarcoma cells in each groin. Unheated serum from the same lot proved notably inhibitory, however, and so too did a specimen that had been heated at 56°C. for 30 minutes, though the latter was somewhat less effective than the unheated material.

Fractionation Experiments.—In each of three experiments the effective constituent was present in materials precipitated from whole guinea pig serum by means of ammonium sulfate in molar concentrations of 2.5, 2.4, and 2.0 respectively. In one of the tests, the findings of which are set down in Chart 10, the material precipitated from guinea pig serum with ammonium sulfate at a molar concentration of 2.4 (pH 5.3) was notably inhibitory when injected intraperitoneally in mice that had been implanted 1 hour before with lymphosarcoma 6C3HED cells, while that separated from rabbit serum under the same conditions had no effect.

The results of four additional fractionation experiments showed that the inhibitory material regularly remained in solution when whole guinea pig serum was dialyzed against 0.02 molar phosphate buffer at pH 5.3 according to a slight modification of the method employed by Ecker *et al.* for the separation of the "midpiece" and "endpiece" components of complement from the serum of human beings (4). In addition to the inhibition tests, electrophoretic analyses were made of the two fractions, a modification of the filter paper method of Flynn and De Mayo being employed (5).[1] These analyses showed that the midpiece fractions, which had been redissolved in 0.9 per cent NaCl solution buffered at pH 7.3, were comprised of components having electrophoretic mobilities corresponding with those of the gamma, beta, and alpha

[1] Dr. Goetz Richter generously made the electrophoretic analyses here mentioned and those in the succeeding experiments as well.

Chart 9

Effects of heat on the inhibitory constituent of guinea pig serum

Experimental Groups*	Mouse No.	Side	Day 7	Day 8	Day 9	Day 10	Day 12	Day 14	Day 16	Day 20
1) Mice Injected Intraperitoneally with 2.0 cc. of Ringer's Solution	1	L	N	●	○	N	N	N	N	N
		R	●	●	○	N	N	N	N	N
	2	L	●	●	●	●	●	●	●	●
		R	●	●	●	●	●	●	●	●
	3	L	●	●	●	●	●	●	●	●
		R	●	●	●	●	●	●	●	●
	4	L	●	●	●	●	●	●	●	●
		R	●	●	●	●	●	●	●	●
2) Mice Injected with Unheated Guinea Pig Serum, Diluted 1:2 with Ringer's Solution	5	L	N	N	N	N	N	N	N	N
		R	N	N	N	N	N	N	N	N
	6	L	N	N	N	N	N	N	N	N
		R	N	N	N	N	N	N	N	N
	7	L	N	N	N	N	N	N	N	N
		R	N	N	N	N	N	N	●	●
	8	L	N	N	N	N	N	N	●	●
		R	N	N	N	N	N	●	●	●
3) Mice Injected with Guinea Pig Serum that had been Diluted 1:2 with Ringer's Solution and Heated at 56°C. for 30 min.	9	L	N	N	N	N	N	N	N	N
		R	N	N	N	N	N	N	N	N
	10	L	N	N	N	N	●	●	●	●
		R	N	N	N	N	N	N	N	●
	11	L	N	N	N	N	●	●	●	●
		R	N	N	N	N	●	●	●	●
	12	L	N	N	N	N	●	●	●	●
		R	N	N	N	○	●	●	●	●
4) Mice Injected with Guinea Pig Serum that had been Diluted 1:2 with Ringer's Solution and Heated at 66°C. for 30 min.	13	L	●	●	○	N	N	N	N	N
		R	●	●	●		N	N	N	N
	14	L	●	●	●	●	●	●	○	N
		R	●	●	●	●	●	●	●	N
	15	L	●	●	●	●	●	●	●	●
		R	●	●	●	●	●	●	●	●
	16	L	●	●	●	●	●	●	●	●
		R	●	●	●	●	●	●	●	●

Outcome of Implantations — Days following Implantation

* All mice implanted with 2.5 million (6C3HED) lymphosarcoma cells in L and R groins.

2.0 cc. of each serum specimen was injected into the peritoneal cavity of the mouse 1 hour after the implantations.

CHART 10

Effects of a fraction of guinea pig serum precipitated with 2.4 M ammonium sulfate, pH 5.3

Experimental Groups*

Outcome of Implantations

Days after Implantation
9 11 13 15 18 20

Mouse No.

1) Control Mice given 0.9% NaCl Solution

1 - L R

0 2cm

2 - L R

3 - L R

2) Mice given "Insoluble Fraction"** of Guinea Pig Serum Precipitated with 2.4M Ammonium Sulfate

4 - L N N N N N N
R N N N N N N

5 - L N N N N N N
R N N N N N N

6 - L N N N N N N
R N N N N N N

3) Mice given "Insoluble Fraction"** of Rabbit Serum Precipitated with 2.4M Ammonium Sulfate

7 - L R †

8 - L R

9 - L N
R

* All mice implanted with 2 million lymphosarcoma cells (6C3HED) in L and R flanks.

** The fractions were precipitated by adding 14 volumes of cold 2.4 M ammonium sulfate, with solution of the precipitate in buffered 0.9 per cent NaCl solution, pH 7.3, and dialysis against the same according to the method of Ecker and Pillemer (*J. Biol. Chem.*, 1940, 135, 347).

globulins of guinea pig serum in amounts of approximately 0.10, 0.03, and 0.06 gm. per cent, respectively, in the three experiments. Saline solutions of these precipitates were regularly devoid of ability to inhibit growth of Lymphosarcoma 6C3HED cells in repeated tests in which the precipitated material from 4 cc. or 8 cc. of whole guinea pig serum was injected into each test mouse 1 hour after implantation with the standard number of lymphosarcoma cells. By contrast, the soluble (endpiece) fractions were always inhibitory, being approximately equal in this respect to whole guinea pig serum. Electrophoretic analyses indicated that the endpiece fractions contained materials having mobilities comparable with those of the various components of whole guinea pig serum, though the albumins were relatively greater in amount, while the globulins were proportionately less, the separation of the various components of the alpha and beta fractions being insufficient to warrant classifying them individually.

Two further attempts were made to isolate the active material by fractionation procedures. Since the endpiece fractions had regularly contained the active material in the experiments previously done, these were used as starting materials. Chart 11 shows the results of electrophoretic and chemical analyses to determine the nature of the fractions procured in the two experiments, and of biological tests to determine their ability to inhibit growth of lymphosarcoma cells *in vivo*. From this it can be seen that the electrophoretic patterns, procured by means of the filter paper method, sufficed for the identification of the major components of the serum specimens, though the mobility of the various alpha and beta globulins was again not sufficiently different to permit their classification as such. Although differing notably in composition, the various fractions all proved inhibitory in the biological tests, as the chart shows. Details of the experiment follow.

In Experiment A of Chart 11, an endpiece fraction—*i.e.* the fraction remaining in solution during 18 hours' dialysis in 18 mm. bags against cold 0.02 M phosphate buffer at pH 5.3—was dialyzed a further 18 hours in a special rotation dialyzer against 1.8 M ammonium sulfate, with separation of the "E 1.8 M-insoluble" and "E 1.8 M-soluble" fractions by centrifugation, the insoluble material being next dissolved in 0.9 per cent NaCl at pH 7.3, and both materials then being dialyzed for 48 hours against frequent changes of 0.9 per cent NaCl pH 7.3 to free them from sulfate ions. In experiment B, the endpiece fraction was dialyzed in 18 mm. bags for 4 hours in the rotation dialyzer against cold 2.2 molar ammonium sulfate, again with separation of the precipitate by centrifugation, resolution of it in 0.9 per cent NaCl pH 7.3, and further dialysis of both soluble and insoluble fractions to get rid of the sulfate ions. The fractions were labelled respectively "E 2.2 M-insoluble" and "E 2.2 M-soluble" even though it was obvious from the findings in comparison with those of other experiments that insufficient time had been allowed for the dialysis against 2.2 molar ammonium sulfate to reach equilibrium.

The patterns reproduced in Chart 11 make it plain that the "E 1.8 M-soluble" fraction of Experiment A contained considerable concentrations of all the serum components, though conspicuously less of the beta and gamma globulins than were present in the whole serum,

CHART 11

Electrophoretic analyses and inhibition tests with fractions of guinea pig serum

Fractions	Nature of Fractions as Determined by Filter Paper Electrophoresis		Inhibition of Lymphosarcoma Cells In Vivo**			
	Electrophoretic Patterns	Concentrations of Components*	4:1	2:1	1:1	1:2
Whole Guinea Pig Serum (Experiment A)		gms % Albumins A 3.05 Alpha Globulins α 1.34 Beta Globulins β 0.51 Gamma Globulins γ 0.69 Total 5.59		++++	++++	+
) "E 1.8M-Soluble" 4:1 (Experiment A)		Albumins A 3.41 Alpha Globulins α 1.06 Beta Globulins β 0.14 Gamma Globulins γ 0.28 Total 4.89	++++		±	
) "E 1.8M-Insoluble" 4:1 (Experiment A)		Beta Globulins β 0.40 Gamma Globulins γ 1.30 Total 1.70	++++		++	
) "E 2.2M-Soluble" 2:1 Approx. (Experiment B)		Albumins A 2.62 Alpha Globulins α 0.78 Total 3.40		+++±	±	
) "E 2.2M-Insoluble" 2:1 (Experiment B)		Alpha Globulin α 0.18 Beta Globulin β 0.47 Gamma Globulin γ 1.46 Total 2.11		+++±	+±	±

ɔr methods of preparation of the various fractions, see the text.

The concentrations of the various components were calculated from nitrogen determinations of the materials tested and planimetric measurements :ir electrophoretic patterns.

1.0 cc. of the test material, in dilutions corresponding with those of whole serum as indicated, was injected intraperitoneally into each of 3 test mice 1 hour after the implantation of 2 million lymphosarcoma cells in each groin. ++++ = complete or nearly complete inhibition of growth; -, ++, +, and ± = respectively: marked, moderate, slight, and very slight inhibition as compared with growth in control animals.

ḥole guinea pig serum was included in Experiment B also; the results were essentially the same as with the serum of Experiment A.

while the "E 1.8 M-insoluble" fraction contained only beta and gamma globulins, the former in almost as large a concentration as was present in the whole serum and the latter in almost twice the concentration. The separation was much sharper in Experiment B, the albumins and the bulk of the alpha globulins being present in the "E 2.2 M-soluble" fraction, while the gamma and beta globulins and a relatively small fraction of the alpha globulins were present in the fraction designated "E 2.2 M-insoluble."

All the materials inhibited the growth of lymphosarcoma cells *in vivo*, as the biological tests showed (Chart 11), the whole serum being more potent than any of the fractions. While the biological tests were by no means quantitative, it was obvious from the results that the "insoluble" fraction in each of the two experiments was somewhat more inhibitory than was the corresponding "soluble" fraction, the results having greater significance because the more active fractions were comprised mainly of beta and gamma globulins while being devoid of detectable albumins and containing comparatively little of the alpha globulins, and also less total protein. Both of the "soluble" fractions were also inhibitory, however, as already implied, that of Experiment A containing, in addition to large amounts of albumins and alpha globulins, considerable though relatively small amounts of beta and gamma globulins also, while the "soluble" fraction of Experiment B contained, according to the findings, only albumins and alpha globulins. The latter material, it may here be noted, was the only one amongst those tested in the present work which was inhibitory and at the same time devoid of amounts of beta and gamma globulins readily measurable by means of the filter paper electrophoresis methods here utilized, though the point deserves emphasis in this relation that relatively large concentrations of these serum components are required for detection and analysis by this means.

In the experiments just given the bulk of the active material went along with the gamma and beta globulins when these were precipitated from the endpiece fractions by means of ammonium sulfate, and the globulin fractions exhibited much more activity in relation to their protein content than did either whole serum or the endpiece fractions. Yet it was obvious that the fractionation procedures employed did not sharply separate either the protein components or the active constituent from the guinea pig serums used as starting materials.

Effects of Chymotrypsin.—In two experiments chymotrypsin was added to solutions of purified materials containing the inhibitory constituent (fractions 3 and 5 of Experiments A and B of Chart 11), the mixtures, together with suitable controls, being tested for inhibitory effects after incubation at 37°C. for 6 hours. Table I shows the results of the tests. In both experiments, it can be seen, small amounts of chymotrypsin, incubated in mixture with the purified materials, promptly rendered the latter non-inhibitory.

The heat, fractionation, and chymotrypsin experiments just given all provide evidence but not proof that the active constituent of guinea pig serum is a protein and that it can be freed from extraneous proteins and concentrated, along with the gamma and beta globulins, by means of fractionation procedures. Yet the findings as a whole make it plain that much further work, and probably approaches by other methods also, will be required before the active material can be isolated and identified.

TABLE I

Effects of Chymotrypsin on the Inhibitory Material in Purified Fractions of Guinea Pig Serum

Experimental mixtures	Outcome of tests for inhibition of lymphosarcoma 6C3HED cells in vivo — Three test mice (*a*, *b*, *c*) implanted with each material		
	a	*b*	*c*
1. "E 1.8 M-insoluble fraction,"* 4:1, 4.5 cc. + BGR, 0.5 cc.	++++	++++	+++±
2. "E 1.8 M-insoluble fraction,"* 4:1, 4.5 cc. + chymotrypsin, 10 mg./cc., 0.5 cc.	0	0	0
3. BGR, 4.5 cc. + chymotrypsin, 10 mg./cc., 0.5 cc.	0	0	0
4. "E 2.2 M-insoluble fraction,"‡ 2:1, 4.0 cc. + BGR, 0.4 cc.	+++	++++	++++
5. "E 2.2 M-insoluble fraction,"‡ 2:1, 4.0 cc. + chymotrypsin, 20 mg./cc., 0.4 cc.	0	0	0
6. BGR, 4.0 cc. + chymotrypsin, 20 mg./cc., 0.4 cc.	0	0	0

Purified salt-free chymotrypsin (Schwarz Laboratories) was employed. The solutions of it were passed through Seitz filters immediately before use.

The experimental mixtures were incubated 6 hours at 37°C. before injection intraperitoneally into each of 3 test mice. 1.5 cc. of mixtures 1, 2, and 3 were injected into the test mice, and 1.0 cc. of mixtures 3, 4, and 5.

++++ = complete inhibition: no growth following intraperitoneal injection of test material 1 hour after implantation of 2 million lymphosarcoma cells in each groin.

+++± and +++ = marked inhibition: one or two growths which appeared late and remained smaller than those in the control mice at each charting.

0 = no inhibition: growths precisely like those in control implanted mice.

* = fraction 3 of Experiment A of Chart 11.

‡ = fraction 5 of Experiment B of Chart 11.

Relationship of the Inhibitory Material in Guinea Pig Serum to Hemolytic Complement: Enhanced Effects of Small Amounts of Guinea Pig Serum in Mixture with Immune Rabbit Serum

The generally known fact that guinea pig serum contains high titers of complement gave rise to the question whether this might be related to its ability to induce regression of transplanted lymphomas *in vivo* as demonstrated in the present work.

In several experiments made initially to test the point, numerous specimens of guinea pig serum were heated at 56°C. for 20 or 30 minutes. These were regularly devoid of ability to hemolyze sheep's erythrocytes in mixture with amboceptor, as supplementary tests showed, while their ability to inhibit the growth of 6C3HED lymphosarcomas in mice was always retained, apparently undiminished in most instances while being slightly diminished in others.

Chart 9, already described, provides an example in which heating at 56°C. for 30 minutes brought about a slight but perceptible loss in inhibitory capacity. A number of additional observations also made it seem unlikely that the inhibitory effect was due to hemolytic complement *per se*. For while the several specimens of horse serum here utilized proved devoid of hemolytic activity for sheep cells in mixture with amboceptor, several of the specimens of frozen normal rabbit serum, and the serum from a normal human being also, had considerable hemolytic activity. For example, in mixture with 5 hemolytic units of amboceptor, 0.2 cc. of one of the rabbit specimens gave complete hemolysis of sheep cells in a dilution of 1:32, while two guinea pig serums in concurrent tests gave titers of 1:64 and 1:128. Yet the rabbit serums proved wholly devoid of ability to inhibit the growth of the 6C3HED lymphosarcoma cells in repeated tests. In a further experiment a sample of guinea pig serum was incubated in mixture with an immune precipitate comprised of horse serum proteins and the antibodies produced by means of them in rabbits, this in order to absorb the hemolytic complement; the absorbed guinea pig serum, separated from the precipitate by centrifugation and having only a slight amount of residual complement, as subsidiary tests showed, proved fully as inhibitory for 6C3HED lymphosarcoma cells *in vivo* as did a control sample of unabsorbed serum tested concurrently.

In spite of the indications just given that hemolytic complement and the inhibitory material in guinea pig serum were not identical, it seemed possible that something more might be learned about the nature of the inhibitory material if tests were made of it in combination with immune sera prepared by injecting the lymphosarcoma cells into rabbits. Sera of this sort were therefore prepared in several groups of rabbits by injecting saline suspensions of the lymphosarcoma cells intraperitoneally, and emulsions of the lymphosarcoma cells and Freud's adjuvants—including heat-killed *Myobacterium butryicum*—intramuscularly (6), the 4 animals of each group, along with 4 normal control rabbits, being bled 3 weeks and again 4 weeks after the immunizing injections.

In vitro tests with the immune sera made it plain that the sera were lethal for the lymphosarcoma cells; this was true, however, only when they were used in low dilutions (*e.g.*, 1:4 or 1:16). The immune sera were fully effective *in vitro* after their own complement had been inactivated by means of heating at 65°C. for 20 minutes, while guinea pig serum was not inhibitory at all, as will be brought out further on. Higher dilutions of the immune sera (*e.g.*, 1:64 or 1:128 or more) were regularly devoid of effect upon the lymphosarcoma cells when held in contact with them *in vitro* during 2 hours at 37°C., though when guinea pig serum was added to such mixtures in final concentrations of 1:10 or 1:20 the cells failed to grow upon implantation in susceptible hosts, and microscopic studies of the lymphosarcoma cells—thrown down into pellets according to a standardized technique (7)—showed that they were all necrotic, with coagulated acidophilic cytoplasm and pyknotic nuclei, while the bulk of the cells in pellets from control mixtures remained unchanged.

The results of many *in vivo* tests showed that small amounts of the immune serum (*e.g.*, 0.2 or 0.02 cc.), given intraperitoneally to mice 1 hour or longer after implantation with 6C3HED lymphosarcoma cells, had no inhibitory

effect on the latter, and the same proved true of small amounts of guinea pig serum (*e.g.*, 0.4 or 0.2 cc.). But when, in several experiments, immune serum in amounts of 0.02 cc. or less was injected together with 0.2 cc. or less of guinea pig serum into mice previously implanted with the lymphosarcoma cells, the latter were completely inhibited as a rule, the quantitative relations making it

TABLE II

Inhibition of 6C3HED Lymphosarcoma Cells in Vivo by Mixtures of Immune Rabbit Serum and Small Amounts of Guinea Pig Serum

Experimental mixtures	Outcome of tests for inhibition of 6C3HED lymphosarcoma cells *in vivo* — Four test mice (*a*, *b*, *c*, *d*) implanted with each material			
	a	*b*	*c*	*d*
1. Saline + normal rabbit serum, 0.02 cc.	0	0	0	0
2. Saline + immune rabbit serum, 0.02 cc.	0	0	0	0
3. Saline + guinea pig serum, 0.2 cc.	0	0	0	0
4. Normal rabbit serum, 0.02 cc. + guinea pig serum, 0.2 cc.	0	0	0	0
5. Immune rabbit serum, 0.02 cc. + guinea pig serum, 0.2 cc.	+++±	++++	++++	++++
6. Immune rabbit serum, 0.02 cc. + heated guinea pig serum (56°C., 30 minutes), 0.2 cc.	+++	++++	++++	++++

The normal and immune rabbit serums were diluted 1:4 with 0.9 per cent NaCl solution and heated at 65°C. for 20 minutes immediately prior to use in order to inactivate complement and natural antibodies. In two other experiments ten and fifteen times as much of the immune serum as was here used—*i.e.*, 0.2 cc. and 0.3 cc., respectively,—failed to inhibit 6C3HED lymphosarcoma cells *in vivo* when injected 1 hour after the implantations.

Each mixture was made up to 0.5 cc. with 0.9 per cent NaCl solution and injected intraperitoneally into 4 test mice that had been implanted 24 hours previously with 1.5 million 6C3HED lymphosarcoma cells in each groin.

0 = no inhibition: growths precisely like those in implanted control mice.

++++ = complete inhibition: no growths.

+++± and +++ = marked inhibition: one or two small growths which appeared late and remained smaller than those in implanted controls.

plan that the inhibition resulted, not from a summation of the effects of the two reagents acting separately, but from the ability of the immune serum to enhance by a factor of four or five times the inhibitory potency of the guinea pig serum *in vivo*. Table II shows the results of one such experiment in detail, it being noteworthy that heating the guinea pig serum at 56°C. for 20 minutes did not destroy the ability of small amounts of it to act effectively in combination with the immune serum.

The findings just given led to the supposition that suitable combinations of

immune serum and guinea pig serum might be more effective in bringing about the regression of large 6C3HED lymphosarcomas in mice, or that this result might be effected with smaller amounts of guinea pig serum than those used in the experiments already given (see Charts 1 to 4, for examples). But in several tests, combinations of immune serum in amounts of 0.1 to 0.5 cc. with guinea pig serum in amounts up to 0.9 cc. proved no more effective in inducing the regression of sizable lymphosarcomas than did large doses of guinea pig serum injected alone or in combination with normal rabbit serum, while amounts of guinea pig serum that were too small to bring about regression of the growths when given alone were likewise ineffective when given in combination with the immune serum.

Tests were next made to see whether immune serum would enhance the effects of purified fractions of guinea pig serum that were known to contain the material inhibitory for lymphosarcoma cells.

The "E 1.8 M-insoluble" material of Experiment A of Chart 11, which was comprised of globulins as the electrophoretic analyses showed, was used in two such experiments. In an *in vivo* test with this material, 1.0 cc. of a saline solution, containing the precipitated globulins from approximately 0.5 cc. of guinea pig serum, was given intraperitoneally to each of 3 C3H mice that had been implanted in each groin 1 hour beforehand with 1.5 million 6C3HED lymphosarcoma cells, while 1.0 cc. of another solution containing the same amount of the purified material in mixture with 0.2 cc. of immune serum was likewise given to 3 additional implanted mice. The purified material alone proved only very slightly inhibitory, growths developing in all 3 of the test mice injected with it which, while appearing 1 or 2 days later than did those of the uninjected control animals and hence being slightly smaller at each of the early chartings, grew quite rapidly and soon became as large as the growths of the control mice. The purified material injected in combination with the immune serum (which in the same amount proved devoid of any inhibitory effect whatever in 3 additional implanted mice of the same experiment) was almost completely inhibitory, 2 of the 3 implanted mice injected with it remaining negative throughout the period of observation (16 days) while a single small growth appeared on the 12th day in one groin of the 3rd test animal. In an *in vitro* test made concurrently, the same material, in a concentration approximately four times that used in the *in vivo* test just described, was mixed with normal rabbit serum and immune rabbit serum, respectively, both in final concentrations of 1:100; saline suspensions containing 10,000 cells per c.mm. were then added in equal parts to the serum mixtures, which were next incubated at 37°C. for 3 hours afterwards. When separated from the incubated mixtures by gentle centrifugation, resuspended in Ringer's solution, and implanted into the groins of 3 test mice, the 6C3HED cells that had been exposed to the purified fraction in mixture with normal serum grew quite readily in the 6 implanted sites of 3 test mice, the outcome being comparable to that following implantation of the cells of control mixtures in other test animals. By contrast, the cells that had been incubated with the purified "E 1.8 M-insoluble" material in mixture with immune serum, when similarly separated from the mixture by centrifugation and implanted into the groins of 3 test mice, failed to grow in 2 of the animals and gave rise to small growths that appeared late (on the 14th day) in the remaining one, while other cells that had been incubated with the immune serum alone grew, following separation and implantation, about as did those of the control mixtures already described. Furthermore, a sample of the purified material that had first been heated at 56°C. for 20 minutes and then admixed with the diluted immune rabbit serum, likewise proved inhibitory for the

6C3HED cells in the *in vitro* test, and the same was true of a sample of guinea pig serum diluted 1:4 with saline and heated at 56°C. for 20 minutes.

In the experiments just given the inhibitory effects of small amounts of whole guinea pig serum were notably enhanced, both *in vivo* and *in vitro*, by admixture with immune sera prepared in rabbits with the lymphosarcoma cells as antigen, and the same proved true of a purified fraction of guinea pig serum containing the inhibitory material; furthermore both the whole guinea pig serum and the active fraction of it retained the ability to combine effectively with immune serum after they had been heated at 56°C. for 30 minutes. The findings suggested strongly that the inhibitory material of guinea pig serum might be identical with one or the other of the heat-stable components of complement; but a number of experiments, now to be briefly described, provided evidence that weighs against the possibility.

In two experiments, specimens of whole guinea pig serum were first heated at 56°C. for 20 minutes to destroy the heat-labile components of complement; some of the specimens were then treated with ammonia, and others with hydrazine, under conditions similar to those employed by Pillemer (8) and Heidelberger (9) and their respective associates for the destruction of the heat-stable C4 component of complement, supplementary tests showing that the complementary activity of specimens of unheated guinea pig serum treated in these ways was in each instance destroyed. The ammonia-treated and hydrazine-treated specimens proved quite as inhibitory for lymphosarcoma cells in the standardized *in vivo* tests as did whole guinea pig serum, however, and the same proved true in an additional experiment of a specimen of heated guinea pig serum that had been treated with liquoid in amounts determined at the time to be sufficient to render unheated specimens non-hemolytic, owing presumably to inactivation of the third component of complement.[2]

In essence it is plain from the experiments just described that while the inhibitory effect of small amounts of the guinea pig serum for lymphosarcoma cells was enhanced in the presence of immune serum under certain conditions both *in vivo* and *in vitro*, the findings as a whole did not provide an answer to the question whether the inhibitory material is identical with one or the other of the heat-stable components of complement. For reasons of expediency the larger question of the nature of the inhibitory material had to be left at this unsatisfactory point, for the time being, though certain of the implications of the studies on this theme will be referred to again in the discussion.

Necrosis and Resorption of Lymphosarcoma Cells in Situ Following Injection of Guinea Pig Serum in Mice

What happens within the lymphosarcomas when these dwindle and disappear following the injection of guinea pig serum into mice carrying them? It seemed possible that the answer to this question might prove helpful in at-

[2] The liquoid and the directions for its use in the inactivation of C′3 were generously supplied by Dr. Myron A. Leon of the College of Physicians and Surgeons, Columbia University.

tempts to understand the mechanism whereby the regression is brought about. Hence a number of microscopic studies were made of the sequence of events that follows injection of guinea pig serum intraperitoneally into mice with subcutaneous 6C3HED lymphosarcomas.

In essence the histological studies showed that the lymphosarcomas of mice given relatively large amounts of guinea pig serum intraperitoneally manifested within a few hours a smaller proportion of mitotic cells than were present in the growths of untreated or horse serum–treated control mice. Furthermore individual lymphosarcoma cells, scattered more or less at random throughout the growths of the treated animals, one after another rapidly became necrotic and were resorbed, the bulk of the cells dying within 16 to 24 hours under such circumstances, and virtually all within 48 hours (Figs. 2 to 4); the further fact was noteworthy that there was little or no inflammatory reaction within the dwindling growths until the bulk of the cells had already become necrotic, while even then the inflammation was often minimal (Figs. 2 to 4). When smaller doses of guinea pig serum were given repeatedly at daily intervals, the same processes followed, but more gradually, so that the diminution in the number of mitotic cells was less striking, while a smaller proportion of necrotic cells and a correspondingly larger proportion of intact lymphosarcoma cells were to be seen in sections of the growths as these gradually became smaller. Indeed numbers of the unchanged cells, some undergoing mitosis, were to be seen during periods of several days following the first of daily injections of relatively small doses of the serum, though under these circumstances the end stages of the process were reached within 4 to 5 days (Figs. 5 and 6). Histological studies were also made of a number of normal organs—lymph nodes, thymus, spleen, bone marrow, lung, liver, kidneys, heart muscle—procured from mice in which lymphosarcomas were regressing following the injection of guinea pig serum; the fact seemed especially noteworthy that these were unchanged. Some of the implications of the histological observations will be considered in the discussion; meanwhile a more systematic description of them will be given.

In the first of two experiments made to learn about the changes that follow promptly after the injection of relatively large amounts of guinea pig serum into mice with lymphosarcomas, 12 female mice, discarded from the breeding colony, were implanted with approximately 5 million 6C3HED lymphosarcoma cells in each groin. The animals were then divided into 3 groups of 4 mice each. One group was kept untreated as controls, while the mice of a second control group were later given horse serum as will be described further on, and those of the third group were given an equal amount of guinea pig serum. At the time of the first serum injections—that is to say, on the 5th day after implantation with the lymphosarcoma cells—firm, rubbery, subcutaneous growths measuring 6 to 9 mm. across were present at both implantation sites in all the mice.

All the animals were in due course killed by decapitation, and small blocks of tissue from the implantation sites in each groin were fixed in acid Zenker; the blocks were then embedded in paraffin, cut at 4 μ, and stained with methylene blue and eosin according to routine pro-

cedures. Scrutiny of these preparations made it plain that the growths in the 4 untreated control animals were characteristic 6C3HED lymphosarcomas, comprised of moderately large spherical cells which on the whole resembled quite closely the large immature lymphocyte of the mouse (Fig. 1). These cells infiltrated the spaces in between the fat cells in characteristic manner and eventually replaced the latter, forming tumors made up of closely packed cells with but scanty stroma and vascular supply (Fig. 1). The "resting" cells generally were quite uniform in size, shape, and staining properties, while those in mitosis were also much alike and very numerous, perhaps 1 in 20 to 1 in 50 of the cells in the growths of untreated mice exhibiting one or another of the division stages at any given time. Here and there amidst the multitudes of living tumor cells comprising the growths of untreated mice, a necrotic lymphosarcoma cell, usually shrunken and distorted, with pyknotic or karyorrhexic nucleus and acidophilic coagulated cytoplasm, was seen. Occasionally in large tumors necrotic lymphosarcoma cells were present in small clusters, especially at the edges of the expanding growths and in areas where hemorrhage was also present, but within the growths the necrotic elements were almost always separated from one another by hundreds of the living lymphosarcoma cells. Inflammatory cells were virtually absent from the growths of the untreated mice, though rarely an isolated neutrophil, mast cell, histiocyte, or lymphoid cell was seen.

The growths of the 4 mice killed 4, 8, 12, and 21 hours after receiving one or two injections of horse serum were precisely like those of the untreated animals, being comprised of large masses of closely packed and more or less uniform and characteristic lymphosarcoma cells, of which approximately 5 per cent in each growth were in mitosis and fewer than 0.5 per cent were necrotic. The growths of the mice given guinea pig serum, however, were all very different from the ones just described. In those of the mouse killed 4 hours after a single injection of the 3:1 "fan-concentrated" guinea pig serum, mitotic lymphosarcoma cells were about one-tenth as numerous as in the growths of control animals, while furthermore approximately 5 to 10 per cent of the lymphosarcoma cells were now necrotic, with coagulated cytoplasm and pyknotic nuclei. The necrotic cells occurred singly or in small clusters, and they were scattered more or less at random throughout the growths, though often they appeared to be arranged in fine lines which joined one another irregularly. They were obviously devoid of any constant anatomic relationship to the blood vessels, and there were no inflammatory cells of any sort in association with them. In the growths of the mouse sacrificed 8 hours after the first injection and 4 hours after the second injection of guinea pig serum, mitoses were again relatively infrequent and nearly half the cells throughout the tumors were necrotic (Fig. 3). though in some areas in which the tumor cells were packed quite closely together, the proportion of necrotic to viable cells was much less, being approximately 1:5 or 1:10. Again, inflammatory cells were absent. Approximately 20 per cent of the lymphosarcoma cells in the growths of the mouse killed 12 hours after the first injection with guinea pig serum were necrotic, the character and distribution of the necrotic elements being about as in the growths previously described. The tumors of the mouse killed 21 hours after the first injection of guinea pig serum and 17 hours after the second injection were much smaller than those in the untreated and horse serum–treated control animals killed at the same time, as gross examination disclosed. In some areas virtually all the tumor cells were necrotic, it being noteworthy that in these areas, as in the tumors as a whole, few or no inflammatory cells were to be seen (Fig. 4); in other areas, however, the viable lymphosarcoma cells still outnumbered the necrotic ones, though mitoses were quite infrequent (approximately 1 in 300 cells) and not a few of the lymphosarcoma cells were swollen and pale-staining.

The second experiment was quite similar to the one just described except that a single injection of 2.0 cc. of 3:1 fan-concentrated guinea pig serum was given intraperitoneally to 4 discarded C3H female breeders with 5 day old 6C3HED lymphosarcomas, instead of two

injections as in the previous experiment. The animals, along with untreated and horse serum-treated controls, were killed for histological studies after 16, 24, 40, and 46 hours, respectively. The tumors of the untreated animals, and those of the mice given horse serum, continued to enlarge as in the previous experiment, and when examined histologically they were seen to be characteristic lymphosarcomas with numerous mitoses and very few necrotic cells. The tumors of the mice given guinea pig serum, which had measured approximately 10 mm. across at the time of the serum injections, softened and dwindled during the ensuing 24 hours, and in the 2 animals sacrificed at 40 and 46 hours, respectively, were neither visible with the magnifying glass when the abdominal skin was reflected to expose the groins, nor palpable. Approximately 80 per cent of the lymphosarcoma cells in the growths of the mice killed at 16 hours were necrotic and many of the rest were swollen and pale-staining. Much the same was true in the growths of the animal killed 24 hours after injection of the guinea pig serum, the fact again being striking that inflammatory cells were virtually absent from the growths though necrotic tumor cells were abundant (Fig. 4), while mitoses were exceedingly rare amongst the viable cells remaining. The sites where growths had regressed in the 2 mice sacrificed 40 and 46 hours, respectively, following injection of the guinea pig serum, showed only a small proportion of necrotic tumor cells and a still smaller proportion of viable but swollen and pale-staining lymphosarcoma cells amidst the innumerable small lymphocytes that now largely occupied the sites and greatly distended the lymphatic channels draining them.

In the two experiments just described the inguinal lymph nodes were almost regularly included in the blocks of tissue taken for histological study. These nodes, whether procured from untreated animals with lymphosarcomas, from those given horse serum, or from those given guinea pig serum in which growths were regressing, were always hyperplastic and comprised of innumerable large and small lymphocytes together with numerous reticulum cells and a few plasma cells, none of which displayed cytologic abnormalities. The hyperplastic nodes not infrequently contained lymphosarcoma cells as well, the latter often being numerous in the cortical sinuses and medullary cords as well as in the peripheral sinuses, and the fact seemed particularly noteworthy that in the animals given guinea pig serum some of the lymphosarcoma cells were necrotic, these being scattered more or less at random as in the subcutaneous growths but more often in the peripheral sinuses than in the deeper structures of the nodes.

In a third experiment done in conjunction with that of Chart 3, histological studies were made of the growths of mice given repeated injections of smaller amounts of guinea pig serum. Two groups of 8 C3H mice were employed, the animals of both groups having been implanted with 6C3HED lymphosarcoma cells as were the mice of the charted groups, and having growths of comparable size on the 4th day. The animals of one group were kept untreated as controls, and those of the other group were given one or more injections of 0.5 cc. of guinea pig serum intraperitoneally on days 4, 5, 6, and 7, one or two of the animals of each group being sacrificed daily between the 4th and 9th day for histological study. Microscopically, as already stated, the process of regression as induced in this way was essentially like that which followed the injection of larger amounts of serum, except that it took place more slowly. A diminution in the number of mitotic cells and a more or less generous sprinkling of necrotic cells, together with moderate numbers of lymphocytes, were manifest in the growths of the serum-treated mice killed on the 5th, 6th, and 7th days. The end stages of the process are illustrated in Figs. 5 and 6.

The findings in the three experiments just given were repeatedly confirmed in histological studies made incidentally in several additional experiments. Furthermore, the lymph nodes,

thymus, spleen, heart, lungs, kidneys, and bone marrow from mice with lymphosarcomas that were regressing or had regressed following the injection of guinea pig serum intraperitoneally were examined in numerous instances. These tissues regularly proved within normal limits when studied histologically, and the same has proved true when these organs were procured at various intervals following the injection of guinea pig serum into normal mice. Incidentally it was noted that the livers of a considerable proportion of the larger and older mice from both the C3H and A colonies displayed a conspicuous cytoplasmic vacuolation resembling quite closely that seen by Elman and Heifetz in the livers of dogs kept on low protein, high carbohydrate diets (10); systematic studies made it plain, however, that this change was seen as frequently in untreated control mice as in those given guinea pig serum or horse serum.

The histological studies as a whole made it plain that the 6C3HED lymphosarcoma cells of tumors *in situ* promptly died and were resorbed following the injection of guinea pig serum into mice carrying them. It seemed especially noteworthy that inflammatory cells were absent from the regressing growths during most of the process, becoming conspicuous only at its end. Furthermore, the cells of the normal lymphoid organs of the host mice (lymph nodes, thymus, spleen) were not rendered necrotic but instead became hyperplastic, following injection of the guinea pig serum, while the cells of other organs and tissues (lung, myocardium, liver, kidney, bone marrow) remained wholly unchanged.

Tests for Effects of Guinea Pig Serum on Lymphosarcoma Cells in Vitro

Does the active constituent of guinea pig serum, circulating in the body fluids of the mice into which it is injected, act directly upon the cells of the lymphosarcomas, killing them as shown in the previous section and thus bringing about regression of the growths? Or does the serum constituent somehow act upon the host and alter one or more of its reactions to the transplanted cells, and thus effect the result? Or does the serum act in conjunction with some substance, or in some special set of conditions, provided by the host? As a first step towards answering these questions, tests were made of the viability of 6C3HED lymphosarcoma cells that had been suspended as individuals in buffered glucose Ringer's solution (BGR) and held in contact with guinea pig serum during periods of several hours *in vitro*.

Three batches of pooled guinea pig serum were employed in four such experiments. Mixtures containing the guinea pig serum, either undiluted or diluted 1:2 or 1:3 with BGR, were incubated, along with control mixtures containing horse serum or rabbit serum, at 37°C. for periods of 4 to 6 hours, with stirring occasionally. The lymphosarcoma cells were then thrown down, by means of brief centrifugation at low speed, into a loose pellet in the bottom of the conical tip centrifuge tubes in which they had been incubated; after the supernatant liquid had been poured off and discarded, the lymphosarcoma cells were resuspended in BGR by means of gentle pipetting; they were then tested for viability by implanting them into the subcutaneous tissues of normal C3H mice. Chart 12 shows the outcome of one experiment. From this it will be seen that lymphosarcoma cells that had remained in contact with

CHART 12

Growth of (6C3HED) lymphosarcoma cells following incubation *in vitro* with normal guinea pig serum

Experimental Groups*	Test Mouse No.	Outcome of Implantations
Suspension of Lymphosarcoma Cells (6C3HED) plus Equal Parts of:		Days after Implantation: 8, 10, 12, 14, 16
a) Normal Guinea Pig Serum	1	L, R
	2	L, R
	3	L, R
	4	L, R
b) Normal Rabbit Serum	5	L, R
	6	L, R
	7	L, R
	8	L, R
c) Normal Horse Serum	9	L, R
	10	L, R
	11	L, R
	12	L, R

0 – 1 cm

* All mixtures incubated 6 hours at 37°C., then centrifuged, with resuspension of the cells in BGR for implantation.

guinea pig serum for 6 hours at 37°C. *in vitro* grew readily upon implantation in susceptible mice, and quite as well as had cells that had been incubated concurrently with horse serum or with rabbit serum.

The same result was got in two additional experiments. In one of these, histological preparations were made of the pellets of 6C3HED lymphosarcoma cells that had been incubated 4 hours at 37°C. in mixture with guinea pig serum, rabbit serum, and horse serum, respectively. The bulk of the cells in the three preparations appeared unchanged on microscopic scrutiny, those that had been held in contact with guinea pig serum being precisely like the ones that had been incubated with rabbit serum or horse serum.

In a fourth test of this sort lymphosarcoma cells, freshly suspended in BGR, were deposited in pellets as already described, resuspended in undiluted guinea pig serum, incubated 6 hours at 37°C. in mixture with the latter, again centrifuged and resuspended in BGR, and tested for viability; these too were viable and gave rise to growths in the test mice that appeared as promptly and enlarged as rapidly as did those resulting from other cells that had been held in contact with undiluted rabbit serum in the same experiment.

The findings just given make it plain that the guinea pig serum did not harm the lymphosarcoma cells *in vitro* under the rigorous conditions of the experiments, and they gain in significance when viewed in light of the fact, brought out in the microscopic studies already described, that the process of regression begins within 4 hours after the guinea pig serum is injected into mice with lymphosarcomas. As a whole the findings provide strong presumptive evidence that the guinea pig serum brings about the regression of lymphosarcomas *in vivo* either by acting in conjunction with some material or in some special circumstances provided by the living host or by altering some reaction of the host to the transplanted lymphoma cells.

DISCUSSION

The facts of the present work seem plain, and so too are the limitations of the inferences that can logically be drawn from them. Yet the essential observation—that normal guinea pig serum brings about regression of experimental lymphomas *in vivo*—remains largely if not wholly empirical. For while the results of several experiments show clearly that the guinea pig serum does not harm the lymphoma cells when held in contact with them during many hours *in vitro*, and hence obviously requires adjuvant influences to bring about the regression, the findings as a whole do not show precisely how this is brought about, and they do not fully disclose the nature of the constituent of guinea pig serum which is responsible for the effect.

The findings provide a basis, however, for at least one suggestion as to the mechanism of the regression, namely that some constituent of the injected guinea pig serum may combine with one or more substances provided by the host—a natural isoantibody, for example, which may have attached itself to the tumor cells following their implantation, perhaps along with one or more of the components of complement, also provided by the host—the combination of factors then bringing about necrosis of the lymphoma cells *in vivo*

by means of a reaction more or less analogous to that which takes place when sensitized cells are brought into contact with the various components of complement *in vitro*. Some indication that an immune reaction may here be at work is provided by the specificity of the phenomenon—as illustrated by the fact that the transplanted lymphoma cells of mice given normal guinea pig serum are rendered necrotic, while transplanted sarcoma cells and mammary carcinoma cells are not inhibited and the mouse's own normal cells are not altered—the findings indicating in addition that the lymphosarcoma cells possess special characters or liabilities. The fact that mouse serum lacks one or more of the known components of complement—and hence is largely devoid of hemolytic activity in mixture with amboceptor (11)—gives rise to the suggestion that in the present experiments the injected guinea pig serum may have supplied one or another or several of the components of complement which are absent from normal mouse serum and perhaps might be required for the full implementation of an immune reaction such as that mentioned above. Still another indication that an immune reaction might be involved in the phenomenon here described is provided by the fact that the effects of the guinea pig serum were enhanced *in vivo* by the presence of immune serum prepared by injecting mouse lymphosarcoma cells into rabbits; furthermore, mixtures of guinea pig serum together with the immune serum brought about morphological changes in mouse lymphosarcoma cells *in vitro* which were identical with those characterizing the process of induced regression *in vivo*, though neither serum alone did so,—the findings having added interest because in another experimental setting complement and a specific antibody acting together brought about striking structural changes of a wholly different sort in Brown-Pearce carcinoma cells (7). Since very little indeed is known about the effects of complement *in vivo*, it is all the more tempting to imagine that the complement contained in guinea pig serum might be responsible in part for the regression of mouse lymphomas here described. Yet some of the facts of the present work actually speak against this possibility, while it is obvious, as already stated, that the findings as a whole do not suffice to define either the nature of the active constituent in guinea pig serum or the mechanism whereby it brings about regression of transplanted lymphomas *in vivo*.

Two practical considerations, already mentioned, limited the usefulness of guinea pig serum as a means of inducing regression of experimental lymphomas in the present work. Firstly, relatively large amounts of serum were required (doses equal to one-tenth the animal's weight often being given), while secondly, the inhibitory effect was sometimes only transitory. Yet as a means of controlling malignant lymphomas in living animals the one here described is both highly effective and highly specific. Indeed it is far more effective and far more specific than the ionizing radiations and chemical materials thus far tested in this relation. For the available agents of these sorts exert effects on experi-

mental lymphomas which on the whole are either inconsequential or merely transitory, and all of them prove lethal for the cells of one or another or several of the essential tissues of the host, as numerous investigations have now plainly disclosed (12–18). A theoretical consideration, however, places a further and still more stringent limitation on the significance of the present work. For the experimental lymphomas here employed were transplanted cancers—that is to say, growths comprised of cells that had originated in animals other than their current hosts—and the lymphoma cells were thus obliged to proliferate in each new host in spite of such resistance as might have been offered by the latter. It is conceivable, as mentioned above, that the regression induced *in vivo* by means of normal guinea pig serum may depend in part upon some natural or induced isoantibody, or upon some other type of immune response which is engendered only by alien cells or effective only against them. Under such circumstances, autochthonus lymphomas—that is to say, neoplasms comprised of the hosts' own cells—would not necessarily regress following the injection of guinea pig serum into individuals carrying them. Whether they will in fact do so remains to be learned.[3]

SUMMARY

In an extension of the experimental studies recorded in an associated paper, attempts were made to isolate and characterize the constituent of guinea pig serum responsible for inducing regression of transplanted lymphomas *in vivo*. The active material was precipitated readily from the whole serum, along with some of the globulins, by means of ammonium sulfate in concentrations of 2.0 molar or greater; it withstood heating at 56°C. for 20 or 30 minutes, but was

[3] The question was not tested in the present work because animals with autocthonus lymphomas were not available. Several observations made during the course of the work, however, weigh against the possibility that the induced regression of transplanted 6C3HED lymphosarcomas was related to the resistance manifested against these alien cells by the C3H mice employed. For example, in several instances (see Charts 1 and 4 of Paper I) lymphosarcomas regressed during treatment with guinea pig serum and reappeared after the injections had been stopped, growing continuously thereafter until the death of the hosts. Such recurrences were not observed in the present work following the spontaneous regression of 6C3HED lymphosarcomas, while furthermore once spontaneous regression occurs the host is thereafter usually solidly and permanently resistant to reimplantation with tumor cells of the same sort, as experience with this tumor and with other growths in numerous laboratories has shown. Furthermore, in the present work a number of C3H mice in which implanted 6C3HED lymphosarcoma cells failed to grow owing to the injection of 1 or 2 cc. of guinea pig serum an hour following the implantations, have proved fully susceptible to reimplantations with the lymphosarcoma cells when tested 10 to 15 days later, an outcome which would be inconsistent with any but the most transitory sort of induced immunity. It should be noted in this relation, however, that Lumsden (*Am. J. Cancer*, 1937, **31**, 430; 1938, **32**, 395) and Gorer (*J. Path. and Bact.*, 1937, **44**, 691; 1938, **47**, 231; 1942, **54**, 51) have cited examples of transitory increases in the titers of isoantibodies in animals implanted with cancer cells originating in alien breeds.

inactivated upon heating at 66°C. for similar periods; it was completely inactivated by chymotrypsin in concentrations of 1 or 2 mg./cc. during 6 hours at 37°C. Furthermore, the inhibitory effects of small amounts of the guinea pig serum *in vivo* were enhanced upon admixture with immune sera prepared by injecting the lymphosarcoma cells into rabbits. The facts as a whole suggest that the active material is a protein, and that it may be one or another of the components of complement; yet they do not suffice to establish its identity.

Microscopic studies showed that the cells of subcutaneous lymphomas rapidly died and were resorbed following injections of relatively large amounts of guinea pig serum intraperitoneally into mice carrying them, while similar changes followed more gradually after repeated injections of smaller amounts of guinea pig serum. No changes referable to the guinea pig serum were seen in the normal tissues or organs of mice receiving it.

Mouse lymphoma cells, suspended artificially as individuals in a physiological saline solution, regularly remained viable following incubation *in vitro* in mixture with guinea pig serum during 6 hours at 37°C. The finding provides strong evidence that the regression of lymphomas that follows injection of guinea pig serum *in vivo* is brought about through some reaction in which the guinea pig serum and the host both participate.

Some of the implications of the findings are discussed.

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EXPLANATION OF PLATES

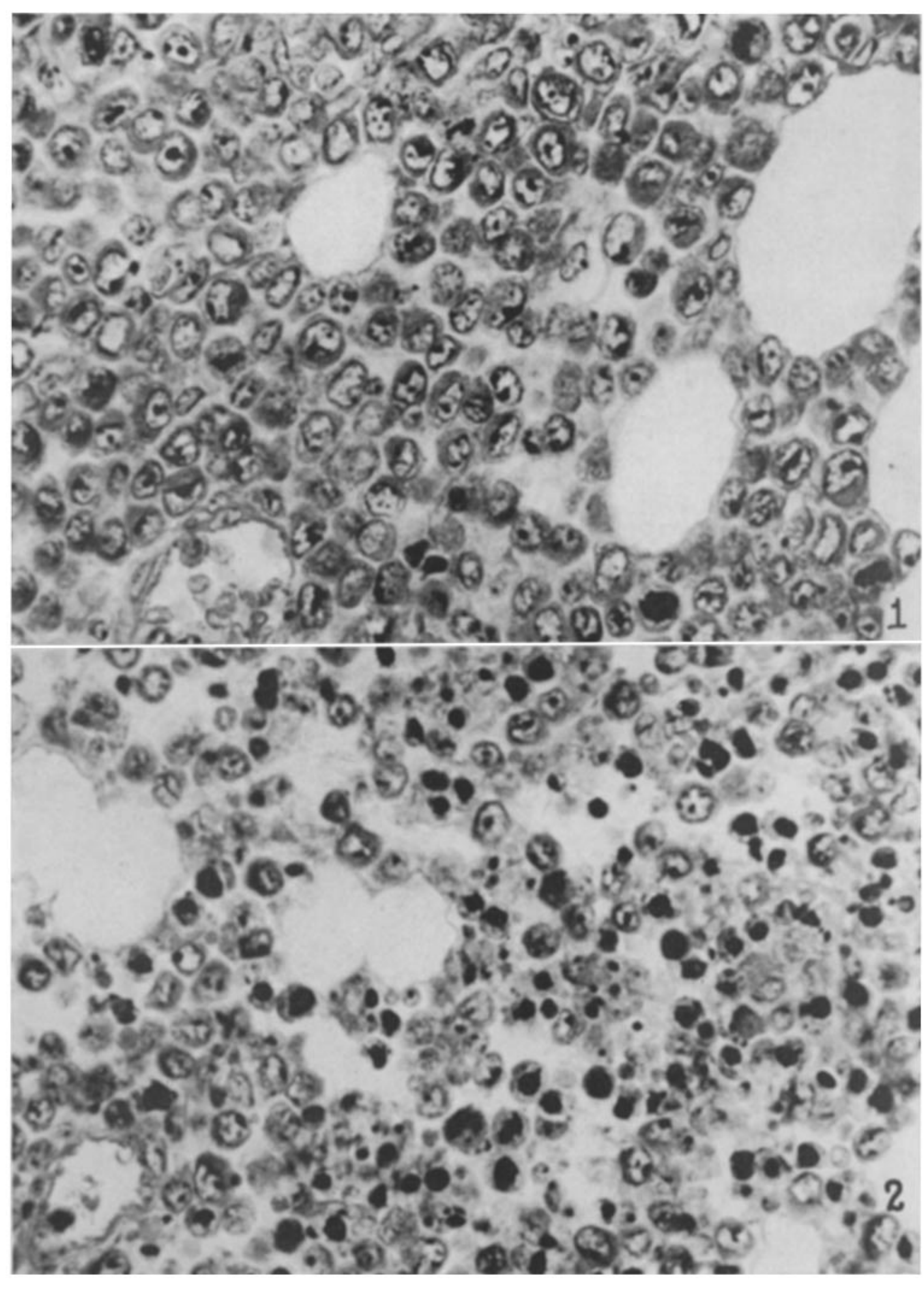
The microphotographs were made by Mr. R. F. Carter. The sections were stained with methylene blue and eosin.

Plate 50

Fig. 1. To show 6C3HED lymphosarcoma cells that were growing in the subcutaneous areolar tissue of an untreated C3H mouse 5 days after implantation. Approximately 5 per cent of the cells throughout the tumor were in mitosis. × 820.

Fig. 2. Necrosis of lymphosarcoma cells brought about in 8 hours by two injections of guinea pig serum into the mouse carrying them.

A C3H mouse with discoid subcutaneous lymphosarcomas 11 mm. in diameter, resulting from implantation with 6C3HED cells 5 days before (same suspension as used in the mouse of Fig. 1) was given two injections 4 hours apart of 1.0 cc. of 3:1 fan-concentrated guinea pig serum intraperitoneally. 8 hours after the first injection the animal was killed for histological study. Approximately half the lymphosarcoma cells throughout the softened tumors were necrotic, with acidophilic coagulated cytoplasm and pyknotic nuclei, while many of the remaining cells were altered, having pale-staining cytoplasm and nuclei with relatively inconspicuous nucleoli and clumped chromatin. Approximately 0.5 per cent of the cells were in mitosis. × 820.

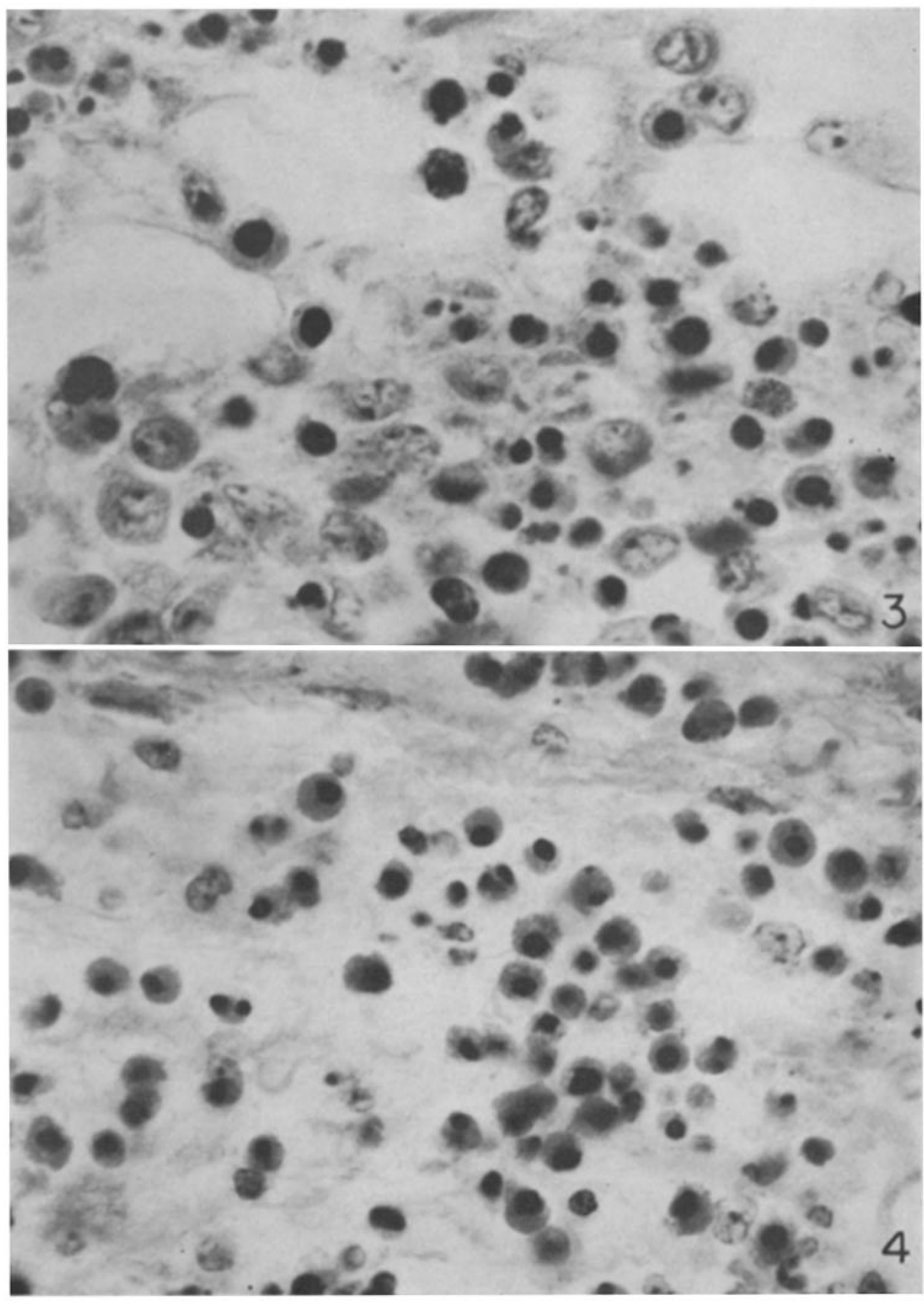


(Kidd: Induced regression of lymphomas. II)

PLATE 51

FIG. 3. A C3H mouse with spherical lymphosarcomas 6 mm. in diameter, resulting from implantations made 5 days previously, was given 1.0 cc. of 3:1 fan-concentrated guinea pig serum intraperitoneally; next day the animal was killed for histological study of its growths, which had undergone marked softening during the 24 hour period. The bulk of the 6C3HED lymphosarcoma cells were necrotic, with coagulated acidophilic cytoplasm and pyknotic nuclei, and nearly all the rest were markedly altered, being swollen and irregularly shaped and having pale-staining cytoplasm, clumped chromatin, and inconspicuous nucleoli. The absence of inflammation is noteworthy. × 1400.

FIG. 4. To show necrotic tumor cells in a 6 day 6C3HED lymphosarcoma that had softened markedly during the 21 hours following the first of two injections of 1.0 cc. of 3:1 fan-concentrated guinea pig serum intraperitoneally into the mouse carrying it. In other areas of the tumor a considerable proportion of the lymphosarcoma cells, although markedly altered, were not yet pyknotic. Again the absence of inflammation throughout the growth was striking. × 910.



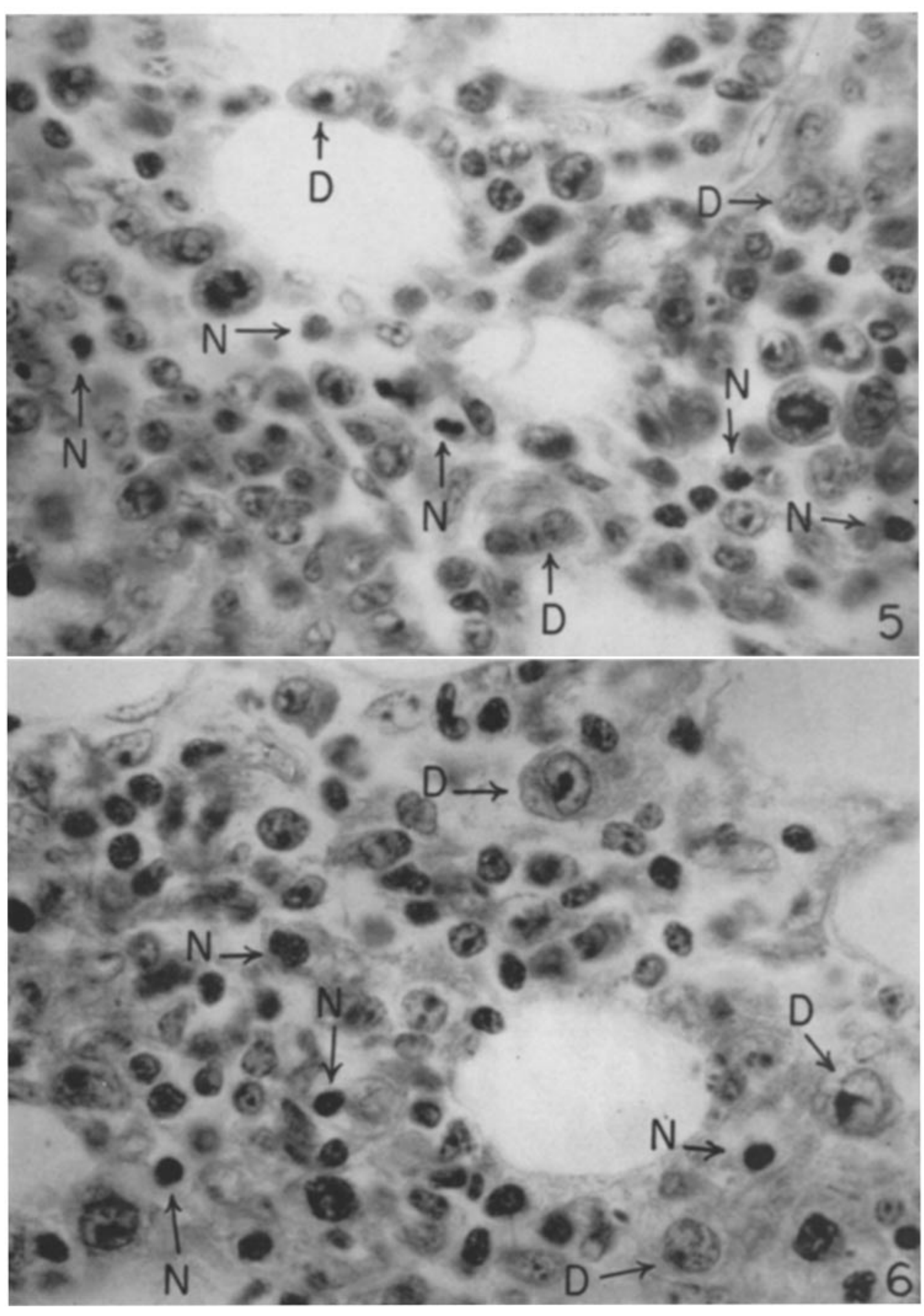
(Kidd: Induced regression of lymphomas. II)

Plate 52

Figs. 5 and 6. To show the effects of repeated injections of small amounts of guinea pig serum.

Fig. 5 shows part of the remains of a lymphosarcoma in a mouse that had been injected with 0.5 cc. of guinea pig serum intraperitoneally on the 4th, 5th, 6th, and 7th days following the implantation of 6C3HED lymphosarcoma cells. The animal was killed for histological study on the 8th day; at this time its growths, which had been 4 mm. across in both groins on the 4th and 5th days, were no longer palpable. Histological studies showed, however, that many lymphosarcoma cells were still present in several small scattered foci in the areolar tissue at the sites of implantation. Some of the lymphosarcoma cells indeed were undergoing mitosis, and two such are shown in the photograph, though the bulk were either degenerating or necrotic. A few cells of the latter sorts are designated in the photograph by arrows marked respectively, *D* and *N*. A number of inflammatory cells with round nuclei are also present. × 1160.

Fig. 6. The animal providing this tissue had been implanted with 6C3HED lymphosarcoma cells as was that of Fig. 5. It was given 0.5 cc. of guinea pig serum intraperitoneally on the 4th, 5th, 6th, 7th, and 8th days, and was killed for histological studies on the 9th day, at which time its growths, which had been 6 mm. across on the 5th, 6th, and 7th days were no longer palpable. A few lymphosarcoma cells were present at the sites of implantation, though most of these were either necrotic (arrows, *N*) or ballooned, with pale-staining cytoplasm and altered nuclei (arrows, *D*); none was in mitosis. Again a few lymphocytes were to be seen. × 1040.



(Kidd: Induced regression of lymphomas. II)