most resistant to atresia (5-10 g) had been developing at an intermediate and slowly declining rate which shows no sharp demarcation from the growth rate of follicles in adjoining weight classes.

Summary. Ovarian follicles in the laying hen were found to exhibit a differential rate of atresia following hypophysectomy which is dependent on follicle size. Atresia appeared first in the smallest of the rapidly developing follicles at 6 hours post-operatively and spread to include all mature or nearly mature follicles by 18 hours. Follicles weighing 5 to 10 g remained intact for as long as 24 hours.

1. Rothchild, I., Fraps, R. M., *J. Clin. Endocrinol.*, 1948, v8, 615.

2. Opel, H., Nalbandov, A. V., *Endocrinol.*, 1961, in press.

3. Warren, D. C., Conrad, R. M., *J. Agri. Res.*, 1939, v58, 875.

4. Smith, A. H., *Poultry Sci.*, 1959, v38, 1437.

Received March 15, 1961. P.S.E.B.M., 1961, v107.

A Thermostable Cytotoxic Factor in Normal Human Serum Active Against Landschutz Ascites Tumor Cells.* (26588)

ITZHAK GINSBURG†, THEODOR DISHON, MARIANNE BLOCH AND JACK GROSS‡
Department of Experimental Medicine & Cancer Research, Hebrew University Hadassah Medical School, Jerusalem, Israel

During previous studies on the cytotoxicity of various agents on Landschutz ascites cells *in vitro*(1,2,3) it was found that fresh normal human serum (NHS) showed a marked cytotoxic effect. Bolande and Todd(4) have reported a similar cytotoxic effect on HeLa cells and Willheim *et al.*(5,6) and Bolande and McClain(7) on Ehrlich ascites cells (EA). According to the latter authors normal human serum contains a thermolabile nondialysable cytotoxic factor active against both Ehrlich cells and Sarcoma 180 cells. They also found that heating to 56°C for 30 minutes and fixation of complement eliminated toxicity for the cells studied.

The present paper reports further information as to the nature of this cytotoxic factor (e.g. that it is stable to 56°C) and as to its mechanism of action (e.g. that it is absorbed by tumor cells and interferes with anti-tumor cell heterologous antibody action).

Materials and methods. Ascites cells (A) were obtained from white mice (local strain) infected with Landschutz ascites tumor. The tumor cells were maintained by weekly passages. Using aseptic precautions, the ascitic fluid was removed 7 days following infection. The cells were washed with Hank's balanced salt solution (BSS) and diluted to a concentration of approximately 5×10^7 cells per ml.

Sera and complement reagents. Pooled normal human serum (NHS) obtained from at least 8 normal donors was stored at −20°C. Serum reagent lacking complement (C′) components C'_1 and C'_2 ($R_1 + R_2$) was prepared by heating NHS at 56°C for 30 minutes which was then designated as NHS 56. Serum reagents lacking properdin (P) $C'_3 + C'_4$ (RP, $R_3 + R_4$) were prepared according to the method described by Kabat and Mayer(8) and Pillemer *et al.*(9). Complement titrations were performed according to the method described by Kabat and Mayer(8) using anti-sheep hemolysin (Difco). C'_{50} unitage of complement was used exclusively.

* Supported in part by the Rose Allen, Irving & Elaine Miller Memorial Club of Chicago, the Florence Kaminsky Cancer Research Club of Chicago, and the Ann Seifer Memorial Club for Cancer Research of Chicago. Presented in part at Symposium on Mammary Cancer Problems, Tel Aviv, Oct. 25-26, 1960.

† At present Magnes Fellow, Research Dept., Children's Hosp., Philadelphia.

‡ This work was carried out during an assignment of one of the authors (JG) as World Health Organization visiting Professor to Hebrew Univ. Hadassah Medical School in collaboration with members of the national staff.

Normal guinea pig (NGPS) and normal rabbit serum (NRS) were pooled from at least 4 animals. The sera were stored at −20°C. In some experiments C′ reagents were prepared from NRS according to the methods mentioned above.

Cytotoxic studies. Washed cells (5×10^6/ml) together with various sera or C′ reagents were made up to a total volume of 1 ml with BSS. The cell suspension was then incubated for 30 minutes at 37°C. At the end of incubation period, 200 cells were counted in each case and the percentage of tumor cells stained with 1% trypan-blue estimated.

Cell suspensions were prepared from the following tissues: Normal human liver, kidney, heart, placenta, amnion, R-III-mouse tumor. (MMClA mammary Ca(14)). The tissue fragments were thoroughly washed with cold BSS, cut into pieces of approximately 1 × 1 mm with scissors and forced through a metal sieve of 80 mesh. The suspensions were centrifuged at 500 rpm for one minute and the supernatant discarded. The sedimented cells were washed twice with 20 ml of BSS and finally diluted to contain 5×10^7 cells per ml. Such cell suspensions usually contained over 50% of dead cells. In some experiments cell suspensions were also prepared from 7 day old cultures of HeLa (Gey) and Cang normal liver cells. The cells were removed from the culture vessel by 0.02% Ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) deficient in Ca^{++} and Mg^{++}; 5×10^6 cells per ml were prepared in BSS.

Fractionation of sera. Serum fractions were prepared by 2 methods, (a) by a modification of Cohn's method(10) and (b) by the Zn-acetate modification of the Cohn technic(11).

Agar electrophoretic studies on the various fractions were performed by a modified method of Aronsson and Gronwald(12) developed in this laboratory using Tris-EDTA-Borate buffer (pH 8.9) applying either 75 V for 16 hours or 130 V for 8 hours. Bromphenol blue was used for visualizing the proteins.

Protein determinations were performed by the tyrosine method of Folin-Ciocalteu(13) using human crystalline albumin as the standard. Readings were performed in a Klett-Summerson colorimeter with filter No. 660.

Anti-Landschutz and anti-HeLa antibodies. Antibodies against ascites cells and HeLa cells were obtained by injecting whole cells in complete Freund's adjuvant subcutaneously into rabbits. Ten days after the second injection, the rabbits were bled from the marginal ear vein and the serum titrated against the cells for cytotoxic activity. A titer of 1:30/ml for 100% death was found, and both cell types underwent rapid cytotoxic changes when brought into contact with their respective antibody. The reactions were complement-dependent, as the antisera heated to 56°C lost their cytotoxic activity. This activity could be restored however by addition of NGPS.

Results. Cytotoxic effect of NHS on ascites cells. The cytotoxic activity of NHS, NHS 56, as well as that of various serum fractions on ascites cells is shown in Fig. 1. The cytotoxic activity of NHS can be destroyed by heating to 56°C for 30 minutes (NHS 56). It was also found that cytotoxic activity of NHS could be prevented by EDTA M/1000, heparin 1 μ/ml, and by streptokinase-activated human fibrinolysin, suggesting that complement-like factors are probably involved in the cytolytic reaction(2,15).

These results corroborate those of Bolande and McClain(7) describing the complement-like nature of the cytolytic factor in human serum. It was also found (Fig. 1) that full cytotoxic activity of NHS 56 could be restored by addition of NRS—containing as little as 2 C'_{50} units, but NGPS, having 40 C'_{50}, was without effect. On the other hand NRS failed to activate NHS heated to 63°C for 30 minutes.

NHS 56 could be substituted by the beta globulin fraction of NHS, but not by the Cohn alpha or gamma globulin fractions. NRS heated to 56°C for 30 minutes, failed to activate NHS 56 or beta globulin. These results suggest that thermolabile factors, present in both NHS and NRS and a thermostable factor (SF) present in NHS only, are involved in this reaction, and that the labile factors contributed by NRS are specific for activation of SF. The nature of SF was therefore further investigated.

Relation of SF to complement (C′). Bo-

lande and McClain(7) have shown that the cytotoxic property of NHS depends on presence of all 4 C′ components and that destruction of any of the C′ fractions impaired the cytotoxicity greatly.

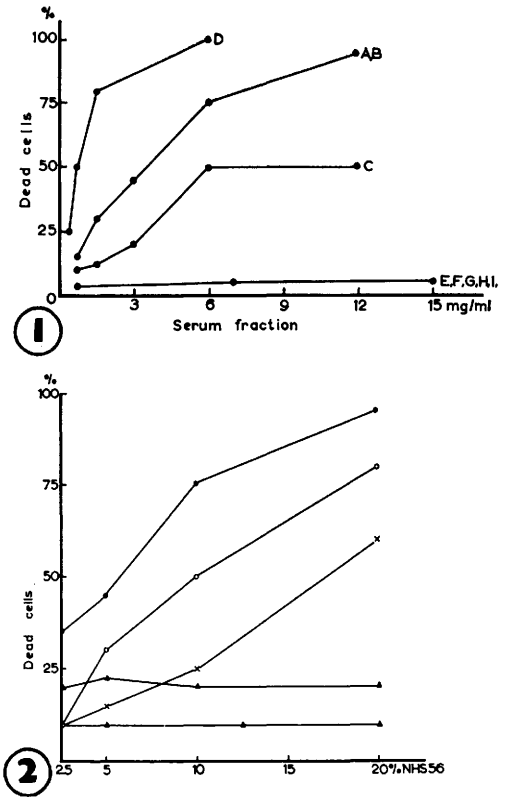

FIG. 1. Effect of normal human serum (NHS) and various serum fractions on Landschutz ascites cells.
A—NHS
B—NHS 56 + NRS 20% ($4C'_{50}$ Hemolytic U.).
C—NHS 56 + NRS 10% ($2C'_{50}$ Hemolytic U.).
D—β Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
E—α Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
F—γ Globulin + NRS 20% ($4C'_{50}$ Hemolytic U.).
G—NHS 56 + NGPS 20% ($20C'_{50}$ Hemolytic U.).
H—Albumin + NRS 20%.
I —NRS.
Serum and serum fractions incubated with A cells for 30 min. at 37°C prior to reading.

FIG. 2. Absorption of NHS 56 on cells.
●——● NHS 56 Control.
NHS 56 absorbed with:
Chang (10^6 cells).
Kidney suspension.
Liver (5×10^6 cells).
Heart suspension.
○——○ Placenta, amnion.
×——× HeLa (3.5×10^5 cells).
△——△ RIII (2×10^6 cells).
▲——▲ Landschutz ascites (5×10^6 cells).
NHS 56 incubated with cell suspensions for 30 min. at 37°C, and supernate assayed for cytotoxic activity on ascites cells in presence of NRS.

TABLE I. Activation of Human Complement Components by NRS.

Reaction mixture	% dead cells*
Ascites cells + NHS 10%†	95
" + NHS 56 10%	3
" + NHS 56 + NRS 20%	95
" + NRS 20%	3
" + NHS 56 R_3 10%	3
" + NHS 56 R_4 10%	3
" + NHS 56 R_3 10% + NRS 20%	80
" + NHS 56 R_4 10% + NRS 20%	25
" + NHS 56 R_3 10% + NHS 56 R_4 10% + NRS 20%	95
" + NHS 56 10% + NHS R_3 10% + NHS R_4 10%	95
" + NHS 56 10% + NRS – R_4 10%	3

* Results were read 30 min. after addition of ascites cells.
† Final concentration in system.

Since NRS could activate NHS 56 which contains only C'_3 and C'_4, the role of both these components in the cytotoxic reaction was determined. R_3 and R_4 reagents of C′ were prepared from NHS 56 and employed in cytotoxicity tests in presence of NRS. Table I shows that NRS added to NHS 56 R_3 caused similar cytotoxicity to that obtained with NHS 56. On the other hand, removal of C'_4 (R_4) markedly reduced its cytotoxicity in presence of NRS. This suggests that the SF has properties similar to the C'_4 of complement. Destruction of C'_4 of NRS by NH_4OH (NRS.R_4) resulted in no cytotoxic activity in the NHS 56 system.

Absorption experiments (Table II). When NHS or NHS 56 were incubated with ascites cells, even at 4°C, SF activity of the serum disappeared from the supernate, after removal of the cells by centrifugation. SF activity was however demonstrable on the absorbing cells, since cell death followed addition of NRS. On the other hand NRS incubated with ascites cells retained its full capacity to activate NHS 56 showing that the NRS activator is not absorbable on tumor cells.

Under the same experimental conditions when NHS was absorbed with cells at 4°C the supernatant failed to kill fresh cells or to activate NHS 56. These results would indi-

TABLE II. Effect of Absorption on Cytotoxic Activity of NHS.

Reaction mixture	% dead cells
NHS 56 10% + NRS 20%	70
NHS 56 + A* + NRS	6
NHS 56 + NRS + A†	70

* NHS 56 was incubated at 37°C for 30 min. with 2×10^6/ml ascites cells. Cells were removed by centrifugation and supernatant assayed for residual cytotoxicity.

† Same as (*) employing NRS.

cate that during the absorption process NHS was deprived of C′ components (probably C'_1 and C'_2) since there was activation of NHS 56 by C'_1 and C'_2 (NHS 56 + $NHSR_3$ + R_4, Table I).

This assumption was further investigated in experiments in which NHS or NHS 56 were incubated with immune aggregates (gamma globulin + anti gamma globulin) (16). Table III shows that while immune aggregates completely removed cytotoxic activity from NHS the activity of NHS 56 remained unimpaired, pointing to the role of C'_1 and C'_2 in this process. The difference in activity between NHS and NHS 56 after incubation with immune aggregate is probably due to C_4 destruction in the former (cf. discussion).

Role of cations on SF absorption. The absorbability of SF was not impaired by the presence of EDTA, citrate, or by dialysis of NHS 56 for 36 hours against normal saline containing EDTA or citrate. This shows that metal ions are not essential to this process. However, addition of EDTA to the NHS 56 + NRS system completely abolished the cytotoxic phenomenon, pointing to the fact that cations are involved in activation of the cytotoxic mechanism.

TABLE III. Effect of Complement Fixation on SF.

Reaction mixture	% dead cells
NHS 10%	75
" + immune precipitate* 10%	5
" + immune precipitate + NRS 20%	16
NHS 56 10%	5
" + immune precipitate + NRS	76
" + NRS	90

* BSS — Washed precipitate obtained from gamma globulin-anti gamma globulin rabbit serum.

Affinity of SF to mammalian cells. Bolande *et al.*(4,7) and Fedoroff(17,18,19) have shown that the affinity of the cytotoxic factor in human serum is not confined only to mouse tumors but also to other mammalian cells. We therefore investigated further the affinity of SF to normal and malignant human cells.

Fig. 2 shows that while the cytotoxic activity of NHS 56 was greatly reduced upon absorption on ascites, mouse mammary carcinoma and HeLa cells, no such phenomenon was observed when Chang normal liver cultures, human kidney, liver or heart cells were used. Thus the malignant cells showed a greater affinity to the SF than these normal cells.

Inhibition of heterologous antibodies against ascites and HeLa cells. Effect of SF. Fedoroff(17,18,19) showed that while human serum obtained from schizophrenic patients was toxic to mouse fibroblasts (L-strain) it was not toxic to HeLa cells, grown in a medium containing human serum. Similarly in our experiments HeLa cells, grown either in presence of human or horse serum could absorb the stable factor but were not killed. The possibility that HeLa cells coated with SF would alter their susceptibility to cytotoxic mechanisms was therefore investigated.

Washed suspensions of ascites and HeLa cells were preincubated with SF for 30 minutes at 37°C. The cells were then washed with BSS and both cell suspensions were incubated with the corresponding heterologous antibodies.

System No. 1 contained ascites cells + anti-ascites serum 56 + NGPS. NGPS was employed in this system because it could not activate NHS 56 but supplied the C′ for anti-ascites serum 56.

System No. 2 contained HeLa cells and their corresponding antibody.

Table IV shows that preincubation of both types of cells with SF markedly reduced their susceptibility to heterologous antibody. These experiments suggest that SF blocked specific receptor sites on the cells, formerly susceptible to the heterologous antibody and complement.

Discussion. A thermolabile complement-like cytotoxic factor in human serum has been

TABLE IV. Inhibition of Cytotoxic Antibodies by SF.

Reaction mixture	% dead cells
HeLa (10^6/ml) + 10% rabbit anti-HeLa serum	100
HeLa + NHS 56 20%	5
HeLa treated with NHS 56 for 30′ at 37°C + anti-HeLa 10%	20
HeLa + NRS 20%	5
HeLa treated with NRS 20% for 20′ at 37°C + anti-HeLa 10%	100
Ascites cells (2×10^6/ml) + 10% rabbit anti-ascites serum heated to 56°C + NGPS	100
Ascites cells + 10% NGPS	5
Ascites cells + 10% NHS 56	6
Ascites cells treated with 10% NHS 56 at 37°C for 30′ + anti-ascites serum 10% + 10% NGPS	30

described by various workers. Fedoroff(17,18, 19) has shown that the serum of schizophrenic patients was toxic to mouse fibroblast cells but had no effect on HeLa cells grown in human serum. Bolande and Todd(4) have described the presence of a factor in pooled human sera toxic to fibroblasts (U 12) and HeLa cells grown on horse serum, but innocuous to normal human fibroblast cultures. The complement components to which this activity was ascribed were C'_3 and C'_4 and probably C'_2.

Bolande and McClain(7) report on a heat-labile cytotoxic factor in human serum, against Ehrlich ascites tumor and Sarcoma 180 cells. This factor was absorbed on the cells, with no inactivation of any of the components of complement. Absorption with the U 12 fibroblasts on the other hand was associated with inactivation of some C'_2 and C'_4.

Kuru *et al.*(20) found that NHS 56 can be activated by NRS, but not by normal marmot serum, to cause the death of Ehrlich ascites cells. We have fully corroborated these findings except for their contention that activity of the SF lies in the gamma globulin fraction of the serum. The evidence in our investigation points to the beta globulin fraction as carrying the cytotoxic activity.

Experiments with the different complement components point to the possibility that a C'_4-like component of C′ might be involved in the cytotoxic mechanism. The fact that NHS after absorption on immune aggregates loses its cytotoxic activity (Table III) further substantiates this assumption(16). C'_1 is a proesterase and is the first C′ component which combines with the antigen-antibody complex. As a result of the combination the proenzyme is activated. C'_4 reacts next, followed by C'_2 and they are irreversibly destroyed by the C'_1 esterase(21). As NHS 56 devoid of C'_1 and C'_2 remains active after absorption on immune aggregates, the possibility exists that SF activity might reside with C'_3 or C'_4. Similarly although Mg^{++} and Ca^{++} are not necessary for absorption of the SF on tumor cells, their presence is an absolute requirement for the cytotoxic reaction, (as is the case for complement activity in general).

The role, if any, of the SF serum component in host resistance to tumor remains to be elucidated in the light of the absorption experiments reported here. A reduction in the serum SF contents in cancer patients *vs* normals, observed in preliminary tests, is now being further substantiated.

Note: After submission of this paper for publication the report of Landy *et al.*(22) became available. It appears likely that the factor described therein is closely similar to the SF reported here.

Summary. A heat stable factor (SF) in normal human serum which is cytotoxic to Landschutz ascites tumor cells has been described. The cytotoxic action requires the presence of human or rabbit complement but guinea pig complement is ineffective. The substance is found in the beta-globulin fraction and has characteristics similar to C'_4 of human complement. It is selectively absorbed on human and mouse tumor cells and human placenta but not by normal human liver, kidney or heart. SF inhibits the action of heterologous antibody on ascites and HeLa cells.

We are indebted to Dr. A. Rimon, Marcus Memorial Blood Bank Institute, Jaffa, for the Cohn fractions, and to Dr. Esther Tenenbaum of this Department for a generous supply of HeLa cells and Chang cells.

1. Ginsburg, I. *Brit. J. Exp. Path.*, 1959, v40, 417.
2. Ginsburg, I., Ram, M., *Nature*, 1960, v185, 328.
3. Ginsburg, I., *Brit. J. Exp. Path.*, 1960, v41, 648.

4. Bolande, R. P., Todd, E. W., *A.M.A. Arch. Path.*, 1958, v66, 720.

5. Willheim, R., Icy, H. C., Janacek, H. M., *J. Exp. Med. and Surg.*, 1957, v15, 300.

6. Willheim, R., Revici, E., Fluss, P., Anber, M., *ibid.*, 1959, v17, 272.

7. Bolande, R. P., McClain, J. P., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 345.

8. Kabat, E. A., Mayer, M. M., In *Experimental Immunochemistry*, 1948, Charles C. Thomas Co. Publishers.

9. Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., Todd, E. W., *J. Exp. Med.*, 1956, v103, 1.

10. Lever, W. F., *et al. J. Clin. Invest.*, 1951, v30, 99.

11. *Blood Cells & Plasma proteins*, Editor J. L. Tullis, Academic Press, Inc., 1953.

12. Aronsson, T., Gronwall, A. *Scandinavian J. Clin. and Lab. Invest.*, 1957, v9, 338.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. I., *J. Biol. Chem.*, 1951, v193, 265.

14. Pikovski, M., Schlesinger, M., *Cancer Research*, 1959, v19, 222.

15. Mayer, M. M., *Progress in Allergy*, 1958, v5, 215.

16. Lepow, J. H., Pillemer, L., *J. Immunol.*, 1955, v75, 63.

17. Fedoroff, S., *J. Lab. and Clin. Med.*, 1956, v48, 55.

18. ———, *Texas Rep. on Biol. and Med.*, 1958, v1, 32.

19. ———, *J. Exp. Med.*, 1959, v109, 615.

20. Kuru, M., Kosaki, G., Terazawa, T., Itouzi, H., Isibasi, H., Tokunaga, A., *Gann*, 1957, v48, 328.

21. Lepow, I. H., Ratnoff, O. D., Levy, I., *J. Exp. Med.*, 1958, v107, 451.

22. Landy, M., Michael, J. G., Trapani, R. J., Achinstein, B., Woods, M. W., Shear, M., *Cancer Research*, 1960, v20, 1279.

Received January 4, 1961. P.S.E.B.M., 1961, v107.

Effect of Brain Hormone from *Bombyx mori* on Metamorphosis of *Calliphora erythrocephala*.* (26589)

MASATOSHI KOBAYASHI AND WALTER J. BURDETTE

Laboratory of Clinical Biology, Department of Surgery, University of Utah College of Medicine, Salt Lake City

Neurosecretion of the brain of insects is known to stimulate the prothoracic gland to function(1-9). Possible direct action of the brain hormone(9) in induction of metamorphosis has been tested in a series of experiments.

Methods.† Mature larvae of *Calliphora erythrocephala*(10) were ligated at the seventh segment and were observed for 24 hr at 25°C. Those with pupation of the segment anterior to the ligature were selected for use in the bioassay. A second ligature was tied posterior to the first and the larva divided between them. After mounting the posterior segment on a glass needle passing through the second ligature, appropriate dilutions of brain hormone and ecdysone, alone and in combination, were injected into respective larval segments. The ligature was useful in closing the anterior end of the specimen after injection and removing it from the needle of the microsyringe. After 24 hours at 25°C, the larval segments were then scored for pupation. Controls included the original bioassay of the brain hormone and ecdysone in which degree of activity was verified in terms of concentration; segments without treatment; and those injected with sesame oil, the solvent for the brain hormone. The results appear in Table I.

Results. Experience with the bioassay indicates that it is qualitative for a single con-

* This investigation was supported by grants from Nat. Inst. Health, Dept. of H.E.W. and Am. Cancer Soc.

† The authors are indebted to Dr. J. Kirimura and Dr. S. Shimizu, Sericultural Experiment Station, Tokyo, for arrangements concerning the preparation of brain hormone. Members of the technical staff of Laboratory of Clinical Biology, University of Utah assisted in preparation of ecdysone, and the technical staff of the Morphological Laboratory, Sericultural Experiment Station participated in preparation of brain hormone.