EFFECT OF STREPTOCOCCAL HÆMOLYSINS ON EHRLICH ASCITES TUMOUR CELLS

I. GINSBURG* and N. GROSSOWICZ

Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(PLATE XXXII)

SEVERAL strains of group-A streptococci possess cell-bound hæmolytic activity (C.B.H.) which can be demonstrated by adding red blood cells (R.B.C.) of various animal species to exhaustively washed streptococcal suspensions in the presence of glucose or other fermentable sugars (Ginsburg and Grossowicz, 1957*a*, 1958). The hæmolytic activity that appears under these conditions is closely associated with the bacterial cells, and no extracellular hæmolysin can be demonstrated. The C.B.H. can, however, be released into the surrounding medium by some surface active agents, e.g., Tween 40, Tween 80 and Triton, and by crystalline albumin.

This hæmolysin was designated as streptolysin D and conveniently abbreviated to S.L.D. (D for detergent). On the other hand, we have failed to release the hæmolysin with sonic energy. Since streptolysin O (S.L.O.) (Todd, 1938; Slade and Knox, 1950) can be easily demonstrated in supernatants, we found no difficulty in differentiating between the two hæmolysins. Moreover, anti-streptolysin O (A.S.L.O.) and cholesterol, which are known to inhibit S.L.O. activity (van Heyningen, 1950) have no effect on C.B.H. or S.L.D.

S.L.D. differs from streptolysin S (S.L.S.) (Todd, 1938; Bernheimer, 1949) in not requiring RNA and maltose for its production by resting streptococci, and by showing a higher resistance to ultraviolet irradiation and to phenylserine than the S.L.S. system (Ginsburg and Grossowicz, 1958). It also appears to be different from the intracellular hæmolysin (I.H.) of Schwab (Schwab, 1956), since sonic energy, which was used by this investigator to release I.H. from streptococci, completely inactivates the C.B.H. A detailed account of the relationship of S.L.D. to other streptococcal hæmolysins is given by Ginsburg and Grossowicz (1958).

Koshimura *et al.* (1955) and Ohta (1957) showed that washed Ehrlich ascites tumour cells lost their invasiveness in white mice if they were previously incubated with washed group-A streptococci of strain S. Incubation of the cells with heated streptococci, streptococcal filtrates, α-hæmolytic streptococci, enterococci, pneumococci, or a variety of other micro-organisms had no such tumour-inhibiting effect. More recently, Koshimura *et al.* (1958) described the formation of streptolysin S by washed streptococci incubated with Ehrlich ascites tumour cells, and asserted that it was produced at the expense of the ribonucleic

* Present address: Department of Experimental Medicine and Cancer Research, The Hebrew University-Hadassah Medical School, Jerusalem, Israel.

acid in the tumour cells; washed streptococci that had not been pre-incubated with tumour cells failed to show any hæmolytic activity. However, no data were included to show whether or not S.L.S. was responsible for the tumour-inhibiting effect described in their experiments.

In the present study the cytotoxic and tumour-killing effect of washed streptococci possessing C.B.H., and of S.L.D., the cell-free form of C.B.H., were studied. The effect of S.L.D., S.L.O., and S.L.S. on various mammalian cells will also be described.

Materials and methods

Streptococcal strains. The following group-A strains were employed: (*a*) strain S84 (type 3), with which most of the experiments described below were carried out, was obtained from State Serum Institute, Copenhagen; (*b*) Richard's strain (type 6, N.C.T.C. London); (*c*) strain C203S (type 3) and (*d*) strain C203U (type 3) were kindly supplied by Dr A. W. Bernheimer, New York University College of Medicine. The strains were grown in brain-heart infusion medium (Difco) or in a complete synthetic medium (Ginsburg and Grossowicz, 1957*b*), in both of which strains *a*, *b* and *c* produced S.L.O. and C.B.H. S.L.S. was also produced by these strains if RNA and maltose were added to resting cells or growing streptococci (Bernheimer, 1949). Strain *d* produced S.L.O. only (Bernheimer, 1954).

Cell-bound hæmolysin (C.B.H.). Tubes containing 10 ml. of brain-heart infusion broth were inoculated with 0·1 ml. of a 12-hr culture of strain S84 and incubated for 12 hr at 37° C. The growth was removed by centrifugation and washed 3 times with 20 ml. of buffered saline, consisting of equal volumes of saline and *M*/15 phosphate buffer, *p*H 7·4. The washed suspension was diluted in buffered saline to contain approximately 5×10^8 cells per ml.; it contained approximately 50 hæmolytic units (H.U.) of C.B.H. per ml. (*v. infra*). To obtain high hæmolytic activity, 1 ml. of a washed streptococcal suspension was incubated for 5 min. at 37° C. in the presence of an "activating mixture" consisting of 500 μg. per ml. each of glucose, $MgSO_4.7H_2O$ and cysteine free base. One ml. of the reaction mixture was serially diluted with buffered saline, and hæmolytic activity was determined by addition of a 1 per cent. suspension of human or rabbit R.B.C. Hæmolytic activity was determined as hæmolytic units per ml. by the method of Slade and Knox (1950). Under the conditions described, 5×10^8 cells were usually found to possess approximately 1000 H.U. of C.B.H. As the possibility existed that washed streptococci still contained traces of streptolysin O, and as cholesterol is known to inhibit S.L.O., but not C.B.H. activity, titrations of C.B.H. were always performed in the presence of 10 μg. per ml. of cholesterol.

Streptolysin D (S.L.D.). To obtain S.L.D. activity, thoroughly washed streptococcal suspensions obtained from a 12-hr culture were incubated for 5 min. at 37° C. in presence of glucose, Mg^{++}, cysteine, and 25 mg. per ml. of salt-free human crystalline serum albumin (Cutter Laboratories, Berkeley, California), and the streptococci were then removed by centrifugation. Under these conditions high hæmolytic activity was found in the supernatant fluid.

S.L.D. was titrated in a manner similar to that used for C.B.H. but with cell-free supernatants instead of cell suspensions. Usually 0·5 ml. of supernatant obtained from 5×10^8 streptococci yielded 1000-2000 H.U. of S.L.D.

Streptolysin O (S.L.O.). As source of S.L.O. the supernatant fluid obtained from strain S84 grown for 18 hr in brain-heart infusion medium was used. To secure maximal activation, 0·5 ml. volumes of the supernatant fluid were incubated for 10 min. at 37° C. in the presence of 1 mg. per ml. of cysteine and

titrated for S.L.O. by the method of Slade and Knox (1950). The supernatant contained approximately 1000 H.U. per ml. of S.L.O. In order to test whether S.L.O. was the only hæmolysin present, supernatant fluids were also tested in the presence of 10 μg. per ml. of cholesterol.

Streptolysin S (S.L.S.). The hæmolysin was prepared and assayed by the method of Bernheimer (1949). Preparations obtained from 5×10^8 streptococci yielded approximately 1000 H.U. of S.L.S.

Experiments with mammalian cells. The following cells were used to determine the effects of streptococcal hæmolysins: (*a*) Ehrlich ascites tumour cells; (*b*) tissue cultures of chick fibroblasts; (*c*) tissue cultures of amnion cells, and (*d*) mouse and rabbit leucocytes.

Tissue cultures were trypsinised with 0·2 per cent. trypsin 1 : 300 (N.B.C.) for 20 min. and washed with buffered saline before use. Mouse and rabbit leucocytes were obtained from animals injected intraperitoneally with 3 ml. of 1 per cent. rice starch; 24 hr later the animals were injected intraperitoneally with buffered saline (5 ml. for mice, 20 ml. for rabbits) containing 1 unit of heparin, and the leucocyte-containing fluid was aspirated with a syringe. All cells were washed three times with buffered saline and kept in ice until used.

In a typical experiment, 0·1 ml. washed mammalian cells (10^8 cells per ml.) were incubated at 37° C. with 0·2 ml. of (*a*) washed streptococcal suspension containing 5×10^8 cells per ml., and possessing 1000 H.U. of C.B.H. activity per ml.; (*b*) S.L.D. solution (1000 H.U. per ml.), released from the streptococci by albumin; (*c*) filtrates containing 1000 H.U. per ml. of S.L.O.; (*d*) S.L.S. solution (1000 H.U. per ml.); and (*e*) streptococci killed by heating at 100° C. for 30 min. After 20-90 min. of incubation 2 drops of 2 per cent. trypan blue solution were added to all tubes, and the percentage of dead cells, which are coloured by the dye, was estimated; usually 500 cells were counted in each experiment.

In addition, various bacterial species were tested for their capacity to affect tumour proliferation in mice. One ml. of washed bacterial suspensions (strain S84, C203S, C203U, *Streptococcus viridans* isolated from dental caries, group-D streptococci isolated from human fæces, or a stock culture of *Lactobacillus casei* 7469) containing approximately 5×10^8 cells per ml. was incubated with 10^7 Ehrlich tumour cells per ml. After 90 min. of incubation at 37° C. 500 units per ml. of penicillin were added to all tubes, and 1 ml. volumes were then injected intraperitoneally into groups of 10 mice weighing 20-30 g. The survival time of both treated and untreated mice was recorded.

Results

Action of C.B.H. and S.L.D. on Ehrlich tumour cells

Table I shows that only living group-A streptococci endowed with C.B.H. activity (strains S84, C203S) could prevent the death of mice harbouring the tumour. Heat-killed strain S84, living streptococci of strain C203U, viridans and group-D streptococci and *Lactobacillus casei*, none of which possessed C.B.H. activity, had no effect on tumour cells, and all mice thus treated died within 10 days following injection of the tumour suspension. As strains S84 and C203S active against tumour cells were endowed with C.B.H. activity despite washing (Ginsburg and Grossowicz, 1958), experiments were set up to see whether the tumour-killing factor was C.B.H.

Table II shows that both C.B.H. and cell-free S.L.D. killed the majority of tumour cells. On the other hand, trypan blue and

aureomycin, which abolish the hæmolytic activity of cell-free and cell-bound hæmolysins respectively (Ginsburg and Grossowicz, 1958) prevented death of the tumour cells. Similar results were obtained with other group-A streptococci possessing C.B.H. activity (C203S and Richard's strains). To prove that the S.L.D. preparations did not contain S.L.S., we have employed albumin extracts from streptococci irradiated with ultra-violet light for 2 min. Such irradiation completely abolishes S.L.S. formation whereas S.L.D. formation is hardly affected (Ginsburg and Grossowicz, 1958).

Morphologically, there was a striking distinction between untreated Ehrlich tumour cells and those subjected to C.B.H. or S.L.D. Untreated tumour cells appeared round with smooth edges. The cell

TABLE I

Survival time of mice injected with Ehrlich tumour cells pre-incubated with various microorganisms

Material injected	C.B.H. activity of bacteria	No. mice dead/no. mice injected, at day	
		10	90
Ehrlich tumour cells	—	10/10	...
Ehrlich tumour cells + strain S84	+	0/10	0/10
Ehrlich tumour cells + strain S84 heated	—	10/10	...
Ehrlich tumour cells + strain C203S	+	0/10	0/10
Ehrlich tumour cells + strain C203U	—	10/10	...
Ehrlich tumour cells + viridans streptococci	—	10/10	...
Ehrlich tumour cells + group-D streptococci	—	10/10	...
Ehrlich tumour cells + *Lactobacillus casei*	—	10/10	...

nucleus was hardly visible and few granules were seen in the cytoplasm (fig. 1); 3-5 per cent. of the cells were dead. Fifteen minutes after addition of the streptococcal suspension (C.B.H.) or of S.L.D. the tumour cells began to swell and the nucleus became visible. In some of the cells the nucleus seemed enlarged and distorted and many glistening granules appeared in the cytoplasm. At the end of the incubation period the cells were at least twice as large as the control cells and many of them possessed pseudopodium-like structures (figs. 2 and 3). In experiments with washed bacteria, a few streptococcal chains were seen attached to the pseudopodia of some tumour cells, but no bacteria were seen within the injured tumour cells. Thus, the integrity of the membrane of the tumour cells seems to have been affected by either C.B.H. or S.L.D. Staining with trypan blue revealed that 100 per cent. of the tumour cells were dead. Cells thus treated completely lost their capacity to proliferate in mice when injected intraperitoneally.

Distinct cytotoxic effects on Ehrlich tumour cells were seen with much smaller doses of C.B.H. or S.L.D. One cytotoxic unit (C.U.50) of either C.B.H. or S.L.D. is defined as the smallest amount of hæmolysin which after 30 min. of incubation at 37° C. causes visible

damage to 50 per cent. of a standard tumour-cell suspension. Under these conditions one cytotoxic unit is roughly equivalent to 20 H.U.

Sera obtained from rabbits immunised against strain S84 and containing approximately 500 Todd units per ml. of antistreptolysin O failed to prevent the morphological changes and the death of the cells.

TABLE II

Effect on Ehrlich tumour cells of incubation with C.B.H., S.L.D., S.L.O., or S.L.S. for 90 min. at 37° C.

Reaction mixtures	Dead tumour cells (per cent.)
Ehrlich tumour cells in buffer	4
Ehrlich tumour cells in buffer+strain S84 (200 H.U. per ml. of C.B.H.)	100
Ehrlich tumour cells in buffer+strain S84 heated at 100° C. for 30 min.	4
Ehrlich tumour cells in buffer+strain S84+25 μg. per ml. aureomycin (to inhibit C.B.H.)	4
Ehrlich tumour cells in buffer+S.L.D. (200 H.U. per ml.) . .	100
Ehrlich tumour cells in buffer+S.L.D. heated at 100° C. for 30 min.	4
Ehrlich tumour cells in buffer+S.L.D.+25 μg. per ml. trypan blue (to inhibit S.L.D.)	4
Ehrlich tumour cells in buffer+S.L.D.+rabbit anti-strain S84 serum	100
Ehrlich tumour cells in buffer+S.L.O. (200 H.U. per ml.) . .	100
Ehrlich tumour cells in buffer+S.L.O.+10 μg. per ml. cholesterol (to inhibit S.L.O.)	4
Ehrlich tumour cells in buffer+S.L.O.+rabbit anti-strain S84 serum	4
Ehrlich tumour cells in buffer+S.L.S. (200 H.U. per ml.) . .	100
Ehrlich tumour cells in buffer+S.L.S. (200 H.U. per ml.)+25 μg. per ml. trypan blue	4

The figures given are the average for at least 10 experiments with different batches of material.

induced by C.B.H. or S.L.D. On the other hand, when C.B.H. and S.L.D. were pretreated with aureomycin (fig. 4) or trypan blue, which neutralise their hæmolytic activity (fig. 5), the capacity of the hæmolysins to affect the tumour cells was greatly reduced. Tumour cells thus treated appeared somewhat swollen, but only about 3-5 per cent. of them were dead; the suspensions were able to kill mice injected intraperitoneally.

Effect of S.L.O. and S.L.S. on tumour cells

Table II shows that S.L.O. and S.L.S. exhibit a tumoricidal effect similar to that described for S.L.D. One C.U.50 of either S.L.O. or S.L.S. was found to be roughly equivalent to 20 H.U. The morphological changes in the tumour cells were essentially similar to those

described above (fig. 2). However, in the case of S.L.O. the appearance of cell damage was more rapid, and was obvious 1-2 min. after addition of the hæmolysin. A.S.L.O. and cholesterol, which had no effect on C.B.H., S.L.D. or S.L.S., completely inhibited the action of S.L.O. (table II). For successful demonstration of the cytotoxic action of S.L.O., the Ehrlich cells have to be thoroughly washed, as ascitic fluid inhibits S.L.O. action; ascitic fluid does not interfere with C.B.H., S.L.D. or S.L.S. action.

Effect of S.L.D., S.L.O. and S.L.S. on various mammalian cells

Rabbit and mouse leucocytes, chicken fibroblasts and human amnion cells were all sensitive to action of S.L.O. and S.L.D. For the

TABLE III

Effect on mouse leucocytes of incubation with S.L.D. and S.L.O. for 90 min. at 37° C.

Reaction mixtures	Dead cells (per cent.)
Leucocytes in buffer (10^7 cells per ml.)	5
Leucocytes in buffer+S.L.D. (200 H.U. per ml.)	100
Leucocytes in buffer+S.L.D. (200 H.U. per ml.) heated at 100° C. for 30 min.	4
Leucocytes in buffer+S.L.D. (200 H.U. per ml.)+25 μg. per ml. trypan blue (to neutralise S.L.D.)	4
Leucocytes in buffer+S.L.O. (200 H.U. per ml.)	100
Leucocytes in buffer+S.L.O. (200 H.U. per ml.) heated to 100° C. for 30 min.	5
Leucocytes in buffer+S.L.O. (200 H.U. per ml.)+10 μg. per ml. cholesterol (to neutralise S.L.O.)	5

The figures are the average of 3 experiments with different batches of S.L.D. and S.L.O.

effect of S.L.D. and S.L.O. on mouse leucocytes see table III; Brittis and Bernheimer (quoted by Bernheimer, 1954) have obtained similar results for S.L.O. action on leucocytes. In our experiments with S.L.S., the hæmolysin was active against Ehrlich tumour cells, but not against leucocytes; other mammalian cells were not tried. Similarly, Matsuda (quoted by Bernheimer, 1954) and Lawrence (1959) failed to show any cytotoxic effect on either leucocytes or on tissue cultures obtained from human skin. The morphological changes induced in these cells by the various hæmolysins were essentially similar to those encountered in tumour cells. Five to fifteen minutes after the addition of the various hæmolysins the mammalian cells showed marked swelling, the nucleus appeared swollen and distorted, and many pseudopodium-like structures surrounded the affected cells; all the cells appeared dead after 90 min. Chicken fibroblasts were much more sensitive to S.L.D. and S.L.O. than the other cells used. Complete disintegration of the fibroblasts

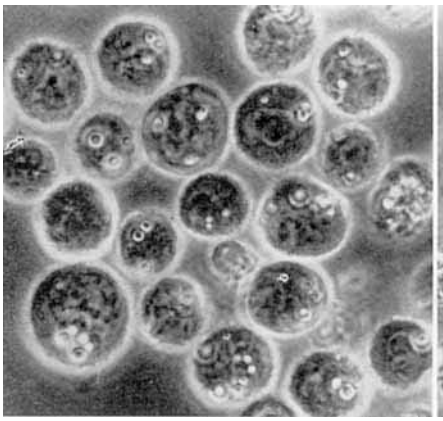

FIG. 1.—Washed Ehrlich ascites tumour cells incubated in "activating mixture" (see Materials and methods) containing 25 μg. per ml. aureomycin, for 90 min. at 37° C.

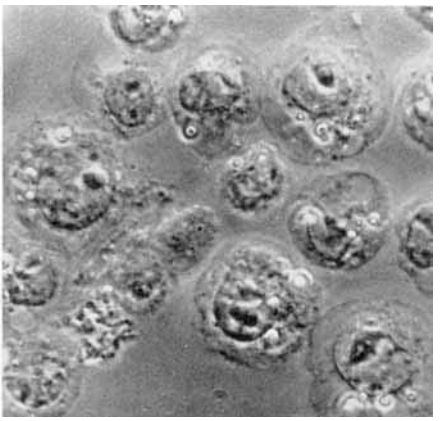

FIG. 2.—Washed Ehrlich ascites tumour cells incubated in "activating mixture" for 90 min. at 37° C. with a washed streptococcal suspension (strain S84 type 3), possessing approximately 200 H.U. per ml. of C.B.H.

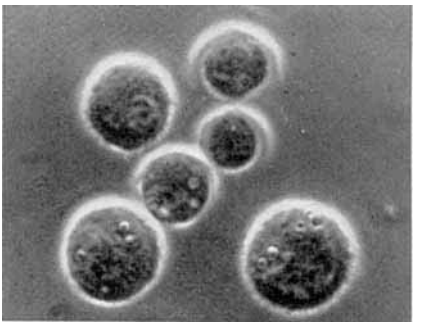

FIG. 5.—Ehrlich ascites tumour cells incubated for 90 min. at 37° C. with 200 H.U. per ml. S.L.D. previously inactivated with 25 μg. per ml. trypan blue.

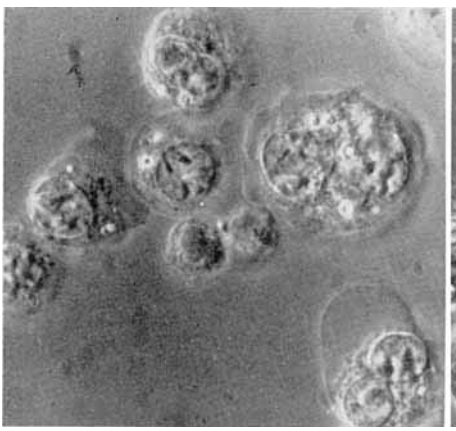

FIG. 3.—Ehrlich ascites tumour cells incubated for 90 min. at 37° C. with 200 H.U. per ml. of S.L.D.

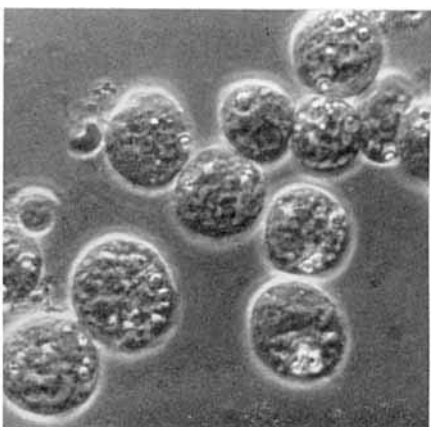

FIG. 4.—Ehrlich ascites tumour cells incubated for 90 min. at 37° C. with strain S84 in "activating mixture" containing aureomycin (25 μg. per ml.). The aureomycin has almost completely inhibited the cytotoxic effect on the tumour cells.

Phase-contrast. ×760.

occurred after 15 min. of contact with either S.L.D. or S.L.O. Thus, as in the case of the tumour cells, the integrity and permeability of the cell membrane of the various normal cells seem to have been affected.

Trypan blue and cholesterol, known to inhibit S.L.D. and S.L.O. respectively, abolished the effect of these hæmolysins on cells.

Discussion

The role of streptococcal infections in tumour regression has been reported by several investigators (Fehleisen, 1882; Coley, 1893). More recently, Havas *et al.* (1958) have described the inhibiting effects of mixed bacterial toxins on sarcoma 37 in mice. These investigators used toxins obtained from streptococcal cultures grown in Neopeptone broth, but they gave no information to suggest to which substances the tumour-inhibiting effects are to be ascribed—to streptolysin O, streptolysin S or any other known streptococcal products.

The data presented here show that S.L.D., S.L.O. and S.L.S. are capable of injuring and killing Ehrlich ascites tumour cells *in vitro*. The morphological changes induced in these cells by action of the various hæmolysins, as expressed by swelling of the cells and pseudopodium-like formation, suggest that, as in the case of red blood cells, these hæmolysins primarily affect the cell membrane and alter its permeability; this causes leakage of intracellular material, leading to the death of the cells. Cells affected by the various hæmolysins completely lost their capacity to proliferate in mice upon intraperitoneal injection. The fact that both tumour and normal cells are similarly affected by S.L.D., S.L.O. and S.L.S. shows that their action is not specific and that under suitable conditions other mammalian cells may likewise be injured.

These results also confirm the findings of Koshimura *et al.* (1955) and Ohta (1957), who have described the action of living group-A streptococci on Ehrlich tumour cells, and the observations of Brittis and Bernheimer (quoted by Bernheimer, 1954), who have shown that S.L.O. is active against mammalian leucocytes.

Moreover, we have succeeded in extracting the active factor from the streptococcal cells with albumin or Tween and have shown that filtrates containing hæmolysins duplicate the effect of streptococcal suspensions. The lethal effect of either C.B.H., S.L.D., S.L.S. or S.L.O. is directly related to its hæmolytic activity. Neutralisation of their activity by trypan blue in the case of S.L.D. and S.L.S., by aureomycin in the case of C.B.H., and by cholesterol and antistreptolysin O in the case of S.L.O., abolishes the cytotoxic properties of these hæmolysins. The experiments with cell-free S.L.D. also suggest that the cytotoxic properties of washed streptococcal suspensions are due to their ability to produce C.B.H., though factors not yet identified may contribute to cytotoxicity. As the tumoricidal action of C.B.H. is a very rapid process, the role of tumour "infection" by the streptococci can be eliminated.

The possible relationship of C.B.H. to the tumour-inhibiting factor present in washed streptococci (Koshimura *et al.*, 1955) is not obvious. However, since group-A streptococci active against tumour cells were the only ones to produce C.B.H., it seems reasonable to suppose that the tumour inhibition obtained by Koshimura *et al.* may have been due to the capacity of the streptococci to produce C.B.H. The inability of streptococcal filtrates to show tumoricidal effect could be explained by lack of the various hæmolysins in the system employed by Koshimura *et al.* (1955). Thus, S.L.D. was probably not present, as there was no Tween or albumin in the medium (cf. Ginsburg and Grossowicz, 1958). S.L.O. was probably absent, as it is not formed by resting cells (Slade and Knox, 1950), and absence of S.L.S. may have been caused by lack of the RNA essential for production of this hæmolysin (Bernheimer, 1949). In a recent communication, however, Koshimura *et al.* (1958) have described the production of S.L.S. by streptococci in contact with tumour cells susceptible to them, and have claimed that the hæmolysin is apparently formed at the expense of RNA of the tumour cells; no data were provided, however, to show that S.L.S. could inhibit tumour proliferation. We, however, have demonstrated that S.L.S. causes distinct cytopathic effects in Ehrlich tumour cells. These apparently conflicting results cannot be explained at present unless it is assumed that the relatively low amounts of hæmolysin present in Koshimura's system (approximately 30 H.U. per ml.) were insufficient to cause severe damage to tumour cells. This assumption seems reasonable, since we have shown that 20 H.U. are roughly equivalent to 1 cytotoxic unit (C.U.50). Moreover, the lack of hæmolytic activity of streptococcal suspensions described by Koshimura *et al.* (1958) might have been due to lack of glucose essential for C.B.H. production, or to rapid deterioration of the cell-bound hæmolysin by incubation at 37° C. for 90 min. as shown in our experiments (Ginsburg and Grossowicz, 1958). Since no data on cytopathic changes were given by Koshimura *et al.* (1955) it is difficult to correlate their findings on tumour inhibition with ours.

Since S.L.D. was produced by 100 out of 116 group-A strains tested, and as several mammalian cells were found to be sensitive to streptococcal hæmolysins, it will be of interest to establish what role, if any, these hæmolytic factors play in the production of cellular damage *in vivo*. Work on these lines is now in progress.

Summary

A cell-bound hæmolytic factor from group-A streptococci, the cell-free form of this hæmolysin (streptolysin D), and the hæmolysin streptolysin O possess cytotoxic activity against Ehrlich ascites tumour cells, mouse and rabbit leucocytes, human amnion cells and chick fibroblasts. Streptolysin S, which was tested against Ehrlich tumour cells and leucocytes, proved active against tumour cells only.

The cytotoxic action of streptolysin D and streptolysin S is not affected by sera from rabbits immunised against group-A streptococci; that of streptolysin O is completely inhibited by antistreptolysin O and by cholesterol.

The morphological changes induced by these hæmolysins, as judged by swelling of cells and by formation of pseudopodium-like structures, suggest that the permeability of these cells has been affected. Tumour cells thus injured lose their capacity to proliferate in mice.

REFERENCES

BERNHEIMER, A. W.	1949.	*J. Exp. Med.*, **90**, 373.
,, ...	1954.	*In* Streptococcal infections, ed. by N. McCarty, *New York*, p. 19.
COLEY, W. B.	1893.	*Amer. J. Med. Sci.*, **105**, 487.
FEHLEISEN, F.	1882.	*Dtsch. med. Wschr.*, **8**, 553.
GINSBURG, I., AND GROSSOWICZ, N.	1957*a*.	*Bull. Res. Counc. Israel*, **6E**, 24.
,, ,, ,, ,,	1957*b*.	*Proc. Soc. Exp. Biol. Med.*, **96**, 108.
,, ,, ,, ,,	1958.	*Bull. Res. Counc. Israel*, **7**, 201.
HAVAS, H. F., GROESBECK, M. E., AND DONNELLY, A. J.	1958.	*Cancer Res.*, **18**, 141.
KOSHIMURA, S., MURASAWA, K., NAKAGAWA, E., UEDA, M., BANDO, Y., AND HIRATA, R.	1955.	*Jap. J. Exp. Med.*, **25**, 93.
KOSHIMURA, S., SHIMIZU, R., MASUSAKI, T., OHTA, T., AND KISHI, G.	1958.	*Jap. J. Microbiol.*, **2**, 23.
LAWRENCE, J. C.	1959.	*Brit. J. Exp. Path.*, **40**, 8.
OHTA, T.	1957.	*Jap. J. Exp. Med.*, **27**, 107.
SCHWAB, J. H.	1956.	*J. Bact.*, **71**, 94.
SLADE, H. D., AND KNOX, GRACE A.	1950.	*Ibid.*, **60**, 301.
TODD, E. W.	1938.	This *Journal*, **47**, 423.
VAN HEYNINGEN, W. E. . . .	1950.	Bacterial toxins, *Oxford*, p. 58.