EFFECT OF STREPTOCOCCAL HÆMOLYSINS ON EHRLICH ASCITES TUMOUR CELLS

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(Plate XXXII)

Several strains of group-A streptococci possess cell-bound haemolytic 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, 1957a, 1958). The haemolytic activity that appears under these conditions is closely associated with the bacterial cells, and no extracellular haemolysin 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 haemolysin 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 haemolysin 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 haemolysins. Moreover, antistreptolysin 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 haemolysin (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 haemolysins 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-haemolytic 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

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acid in the tumour cells; washed streptococci that had not been pre-
incubated with tumour cells failed to show any haemolytic 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,
1957b), 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 haemolysin (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, pH 7·4. The washed suspension was diluted
in buffered saline to contain approximately 5 x 10^8 cells per ml.; it contained
approximately 50 haemolytic units (H.U.) of C.B.H. per ml. (v. infra). To obtain
high haemolytic 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 haemolytic activity was determined by addition of a 1 per cent. suspension
of human or rabbit R.B.C. Haemolytic activity was determined as haemolytic
units per ml. by the method of Slade and Knox (1950). Under the conditions
described, 5 x 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 haemolytic 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 super-
natant obtained from 5 x 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 haemolysin present, supernatant fluids were also tested in the presence of 10 μg. per ml. of cholesterol.

Streptolysin S (S.L.S.). The haemolysin was prepared and assayed by the method of Bernheimer (1949). Preparations obtained from $5 \times 10^8$ 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 haemolysins: (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 $5 \times 10^8$ 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 feces, or a stock culture of Lactobacillus casei 7469) containing approximately $5 \times 10^8$ cells per ml. was incubated with $10^8$ 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 haemolytic activity of cell-free and cell-bound haemolysins 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 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 haemolysin 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.*

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

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 haemolytic activity (fig. 5), the capacity of the haemolysins 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 haemolysin. 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>
<thead>
<tr>
<th>Table III</th>
<th>Effect on mouse leucocytes of incubation with S.L.D. and S.L.O. for 90 min. at 37°C.</th>
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</thead>
<tbody>
<tr>
<td>Reaction mixtures</td>
<td>Dead cells (per cent.)</td>
</tr>
<tr>
<td>Leucocytes in buffer (10⁷ cells per ml.)</td>
<td>5</td>
</tr>
<tr>
<td>Leucocytes in buffer+S.L.D. (200 H.U. per ml.)</td>
<td>100</td>
</tr>
<tr>
<td>Leucocytes in buffer+S.L.D. (200 H.U. per ml.) heated at 100°C. for 30 min.</td>
<td>4</td>
</tr>
<tr>
<td>Leucocytes in buffer+S.L.D. (200 H.U. per ml.) + 25 µg. per ml. trypan blue (to neutralise S.L.D.)</td>
<td>4</td>
</tr>
<tr>
<td>Leucocytes in buffer+S.L.O. (200 H.U. per ml.)</td>
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<td>5</td>
</tr>
<tr>
<td>Leucocytes in buffer+S.L.O. (200 H.U. per ml.) + 10 µg. per ml. cholesterol (to neutralise S.L.O.)</td>
<td>5</td>
</tr>
</tbody>
</table>

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 haemolysin 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 haemolysins were essentially similar to those encountered in tumour cells. Five to fifteen minutes after the addition of the various haemolysins 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. 3.—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. 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 haemolysins 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 haemolysins, as expressed by swelling of the cells and pseudopodium-like formation, suggest that, as in the case of red blood cells, these haemolysins 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 haemolysins 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 Britts 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 haemolysins 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 haemolytic 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 haemolysins. 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 haemolysins 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 haemolysin (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 haemolysin 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 haemolysin 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 haemolytic 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 haemolysin 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 haemolysins, it will be of interest to establish what role, if any, these haemolytic factors play in the production of cellular damage in vivo. Work on these lines is now in progress.

**Summary**

A cell-bound haemolytic factor from group-A streptococci, the cell-free form of this haemolysin (streptolysin D), and the haemolysin 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 haemolysins, 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.

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