The bottles used for in vitro culture were universal containers ('McCartney' bottles). The secrew cap of these bottles was replaced by rubber bung to which were attached glass rods. The glass rods extended to the lower one-third of the bottle. The everted intestine was then slipped on the glass rods. As long as the glass rods were of the right diameter the intestinal segment did not slip off and remained attached to the glass rod. Tissue culture medium which was then added to each bottle consisted of 5–6 ml of medium 199 in BSS (Difco) + 25% fetal calf serum + 25,000 U Penicillin/ml + 25,000 µg Streptomycin/ml. The bottles were placed in a roller drum and allowed to rotate slowly at a speed of about 1 revolution per 2 min. The angle in the tube was such that the fluid covered only one side of the intestine, at any given time, as shown in Figure 1. This allowed alternate exposure of the mucous membrane to the medium and the air in the tube. The medium was changed every 12 h and at the same time examined for the presence of bacterial growth. Only occasionally were the tubes found turbid due to bacterial or fungal growth, which were then discarded. The medium from the clean tubes was centrifuged and the sediment examined for the presence of parasites.

The examination consisted of observations in the light microscope under phase contrast and alcohol fixed Giemsa stained smears. The pooled sediments were also processed for electron microscopy.

The light microscopy showed the presence of merozoites in large numbers up to 3 days of the culture. The merozoites were motile and could be distinguished without any difficulty. Giemsa stained smears showed the morphology of the merozoites clearly. They were crescentic bodies 7–10 µm by 2.5–3.5 µm with a pointed anterior end and a rounded posterior end. The nucleus was ovoid and generally located towards the posterior end (Figure 2). In addition to the merozoites oocysts and macrogametocytes were also observed. All these stages were identical in appearance to the forms described previously (ZAMAN and COLLEY[5]). The number of merozoites fell sharply after 3 days but a few parasites continued to appear up to 7 days.

Sediments made from 12-h-old cultures and examined by electron microscope showed individual host cells containing different stages of parasites. Some of the parasites were degenerating but many were in excellent stage of preservation. As in the light microscopic observation they were predominantly merozoites. Apparently the host cells containing the mature schizonts break off from the mucous membrane and liberate the merozoites in the medium. Figure 3 shows a degenerating host cell containing merozoites. Figure 4 shows a merozoite lying outside the host cell and showing a nucleus and the paired organelles.

The technique described is a simple method for obtaining merozoites of *Toxoplasma gondii* in bacteria free conditions. The bulk of the parasites were obtained during the first 3 days, indicating that the development of the parasite did not continue for a longer time in the medium used. It is, however, possible that further modifications of the medium would enable the parasites to continue their life cycle for a longer period of time.

*Résumé.* Une technique, par laquelle l'intestin du chat infecté par le *Texoplasma* est débarassé des bactéries par l'antibiote et ensuite cultivé dans des éprouvettes est decrivée. Dans ces conditions, on peut obtenir des mérozoïtes en grande quantité durant 3 jours. Les mérozoïtes sont exempts de bactéries et viables. Cette technique peut être appliquée à d'autres espèces de coccidies.

V. ZAMAN and N. B. BHONSALE

*Faculty of Medicine,*
*University of Singapore, Sepay Lines,*
*Singapore 3, 2 December 1970.*

[5] V. ZAMAN and F. C. COLLEY, SEast. Asian J. trop. Med. Publ. Hlth. *1*, 457 (1970).

## In vitro Anti-Tumor Activity of Lipid Extracts from a Group A *Streptococcus* against Ehrlich Ascites Carcinoma in Mice

In 1955, KOSHIMURA et al.[1] reported that washed cells of a group A *Streptococcus*, when premixed with tumor cells before inoculation, completely suppressed the development of ascitic tumors in mice. Their observations have been confirmed and extended by many researchers[2–5]. However, the nature of the factor responsible for the in vitro anti-tumor activity of the organisms has as yet been largely obscure. The present paper gives data on the in vitro anti-tumor effect of lipid extracts derived from a group A *Streptococcus hemolyticus*, strain Su, against Ehrlich ascites carcinoma cells in mice.

Streptococci were grown at 37 °C in 10 l lots of WOOD and GUNSALUS[6] medium. After 12 h incubation, the organisms were harvested by centrifugation and washed thoroughly in saline. The packed cells were then extracted 3 times (twice for 2 h and finally overnight) with 20 volumes of chloroform-methanol (2:1) by stirring at room temperature. The combined extracts were evaporated at 40 °C under a stream of nitrogen. The crude lipid extracts thus obtained were redissolved in chloroform-methanol and washed with saline to remove non-lipid materials as described by FOLCH et al.[7]. Upon concentration followed by drying in vacuo of the chloroform layer, the residual oily mass (total lipids) was brought to a known volume with chloroform-methanol and then fractionated by thin-layer chromatography on plates (20 × 20 cm) covered with silica gel H (1 mm thick), using chloroform-methanol-water (65:25:4)[8] as ascending solvent; the lipid extracts were applied as a narrow band

[1] S. KOSHIMURA, K. MURASAWA, E. NAKAGAWA, M. UEDA, Y. BANDO and R. HIRATA, Jap. J. exp. Med. *25*, 93 (1955).
[2] E. A. CHRISTENSEN, Acta path. microbiol. scand. *46*, 285 (1959).
[3] H. F. HAVAS, A. J. DONNELLY and A. V. PORRECA, Cancer Res. *23*, 700 (1963).
[4] M. H. FERTMANN, B. FERTMANN and A. FURST, Proc. West. pharmac. Soc. *6*, 27 (1963).
[5] H. OKAMOTO, S. SHOIN, S. KOSHIMURA and R. SHIMIZU, Jap. J. Microbiol. *11*, 323 (1967).
[6] A. J. WOOD and I. C. GUNSALUS, J. Bact. *44*, 333 (1942).
[7] J. FOLCH, M. LEES and G. H. SLOAN STANLEY, J. biol. Chem. *226*, 497 (1957).
[8] H. WAGNER, L. HÖRHAMMER and P. WOLFF, Biochem. Z. *334*, 175 (1961).

at the start line of the plate and allowed to develop until the solvent front had migrated about 15 cm. The chromatographed total lipids yielded with iodine vapor[9] six bands, referred to as Fraction, I, II, III, IV, V and VI in the order of the Rf values, 0.08, 0.19, 0.37, 0.51, 0.67 and 0.92, respectively. Qualitative analysis of chemical components of these fractions was carried out by spraying the following reagents; phosphomolybdate[10], ninhydrin, Dittmer and Lester reagent, Dragendorff reagent, anthrone-sulfuric acid, and antimony trichloride. Each of the lipid fractions, being eluted in chloroform-methanol from silica gel, was concentrated and dried in vacuo. For biological testing the lipid fractions were evenly suspended in 0.85% NaCl solution containing 0.02% of Tween 20 so that 1.5 ml of the suspension was the equivalent of 2 g, wet weight, of the coccal cells.

The in vitro anti-tumor effect against Ehrlich ascites carcinoma cells was examined by the method of KOSHIMURA et al.[1], except that phosphate-buffered Ringer solution was replaced with 0.85% NaCl solution. Female mice, 20–22 g, of the *dd* N strain were used throughout. Mixtures of tumor cell suspension ($6 \times 10^7$ cells/ml) with 3 volumes of the different lipid suspensions were preincubated at 37 °C for 90 min. Groups of 10 mice each were then given i.p. inoculation of 0.5 ml of the reaction mixtures, and the survival times were recorded. The experiments were terminated 60 days after inoculation, and all the survivors were sacrificed and examined grossly. In all cases, control experiments were performed in which cell suspensions without lipid fractions were preincubated in saline containing Tween 20 in an amount corresponding to that of the test mixtures. Representative of some of these experiments are summarized in the Table.

In the first experiments whole total lipids from *Streptococcus hemolyticus*, strain Su, were tested for their in vitro anti-tumor activity. As can be seen, mice receiving the tumor cells premixed with whole total lipids at a concentration of 13.5 mg per ml of cell suspension showed no signs of tumors at the end of 60-day-period, while control mice invariably died from ascitic tumors in less than 20 days. Having established the marked activity of whole total lipids, attempts were made to separate the active constituent. Total lipids were fractionated into 6 fractions by thin-layer chromatography as described above and the activity of each fraction was tested against tumor cells at concentrations equal to the level present in the original whole total lipids. It is evident that 2.9 mg of fraction VI per ml of cell suspension completely prevented the development of ascitic tumors. By contrast, no protection was obtained with any one of the other lipid fractions. It is apparent, therefore, that the in vitro anti-tumor activity of the whole total lipids obtained from the cocci was contained almost entirely in the rapidly migrating lipid fraction.

The staining studies of chromatographed lipid fractions established that fraction VI contained neither carbohydrates nor amino acids, but also it could not be phospholipids because of negative reaction to DITTMER and LESTER[11] reagent. The results of these qualitative analyses indicate that the anti-tumor activity is associated with non-polar lipid fraction making up a relatively large part (22.2%) of the total lipids. Further, it is worthy of note that fraction VI stained with antimony trichloride as a light violet spot[12], suggesting the possible presence of sterol or its esters. Our present knowledge about the streptococcal lipids[13] is far from sufficient to understand the chemical constitution of this active non-polar lipids. Nevertheless, considering the complexity in constitution of non-polar lipids between different species of bacteria[14], it is reasonable to assume that fraction VI contains more

[9] R. P. A. SMIS and J. A. G. LAROSE, J. Am. Oil Chem. Soc. *39*, 232 (1962).
[10] H. P. KAUFMANN, Z. MAKUS and F. DIECKE, Fette Seifen *63*, 235 (1961).
[11] J. C. DITTMER and R. L. LESTER, J. Lipid Res. *5*, 126 (1964).
[12] H. WEICKER, Klin. Wochschr. *37*, 763 (1959).
[13] M. COHEN and C. PANOS, Biochemistry *5*, 2385 (1966).
[14] M. KATES, Adv. Lipid Res. *2*, 17 (1964).

In vitro anti-tumor effect of lipids extracted from a group A *Streptococcus hemolyticus*, Su, against Ehrlich ascites tumor in mice

| Lipids extracted from *Streptococcus hemolyticus*, Su | | | | | | | | | In vitro anti-tumor activity | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Staining behaviors[a] | | | | | | | | | |
| Fraction No. | Yields: mg/10 l culture (%) | Iodine vapor | Phospho-molyb-date | Nin-hydrin | DITTMER and LESTER[11] reagent | Dragen-dorff reagent | An-throne-sulfuric acid | Anti-mony trichlo-ride | Concen-tration in test mixture (mg/ml cell suspension) | Average survival (days) | No. of survivors at 60 days |
| Total lipids | 270 | | | | | | | | 13.5 | 60[b] | 10/10 |
| | | | | | | | | | Cell control | 17.4 | 0/10 |
| I | 32 (11.9) | w | w | m | – | – | – | – | 1.6 | 20.5 | 0/10 |
| II | 23 (8.5) | w | w | – | tr | – | tr | – | 1.1 | 17.5 | 0/10 |
| III | 37 (13.7) | s | s | – | s | – | – | – | 1.8 | 21.9 | 0/10 |
| IV | 54 (20.0) | s | s | – | – | w | s | – | 2.5 | 22.5 | 0/10 |
| V | 50 (18.5) | s | s | – | m | w | s | – | 2.5 | 22.0[c] | 1/10 |
| VI | 60 (22.2) | m | m | – | – | – | – | m | 2.9 | 60 | 10/10 |
| | | | | | | | | | Cell control | 18.0 | 0/10 |

[a] Abbreviations: s, strong; m, medium; w, weak; tr, trace; –, negative. [b] Represents survival beyond the 60-day test period. [c] Calculated from times of death of 9 out of 10 mice which failed to survive.

than one lipid component. More detailed studies along this line are now in progress.

From the foregoing it is apparent that it has been possible to obtain from the cell bodies of the group A streptococcus a non-polar lipid fraction highly active in suppressing the development of ascitic tumor when pre-incubated with tumor cells before inoculation. Although it cannot be said with certainty that this non-polar lipid fraction represents the sole component responsible for the anti-tumor activity of the group A *Streptococcus* demonstrated by KOSHIMURA et al.[1], the present results are of special interest in view of the earlier investigations that the lipid preparations derived from royal jelly[15] and *Sh. flexineri* and *E. coli*[16] have been capable of inhibiting the development of experimental tumors in animals. The possible importance of the streptococcal lipids as anti-tumor agents remains to be evaluated.

*Résumé.* On a isolé chez la souris, d'une souche de *Streptococcus hemolyticus* une fraction lipidique qui inhibe complètement le développement de la tumeur ascitique d'Ehrlich quand la fraction lipidique est préincubée avec les cellules de la tumeur avant inoculation.

S. KIGOSHI

*Department of Pharmacology, School of Medicine, Kanazawa University, Kanazawa (Japan), 9 January 1971.*

[15] G. F. TOWNSEND, J. F. MORGAN and B. HAZLETI, Nature, Lond. *183*, 1270 (1959).
[16] N. KASAI, Y. AOKI, T. WATANABE, T. ODAKA and T. YAMAMOTO, Japan. J. Microbiol. *5*, 347 (1961).

## Transfer of R-Factor Mediated Aminoglycoside Antibiotic Resistance in the Allantoic Cavity of Chick Embryos

The discovery of R-factor (episome)-mediated, transferrable, multiple-drug resistance among various species of *Enterobacteriaceae* and *Pseudomonadaceae*[1] prompted several investigators to examine a variety of laboratory animals with regard to their suitability for in vivo transfer of multiple-drug resistance[2–8]. Generally, germ-free animals, infantile animals, or antibiotic-treated-modified conventional adult animals had to be utilized for these experiments to succeed. Reported also was transfer of multiple-antibiotic resistance from *Escherichia coli* of animal or human origin to resident *E. coli* within the gastrointestinal tract of a human volunteer, following oral administration of drug-resistant donor organisms[9]. Here we wish to report in vivo transfer of R-factor-mediated aminoglycoside antibiotic resistance from 2 clinical enterobacterial isolates to a drug-sensitive, recipient strain of *E. coli* within the chick allantoic cavity.

Multiple drug-resistant isolates *E. coli* 1531 and *Klebsiella pneumoniae* 829, from clinical sputum specimens[10], served as donors; the recipient in all experiments was *E. coli* K-12, strain 1485 ($F^-$ $lac^+$), resistant only to nalidixic acid (*E. coli* 1485-Na-R). Stock solutions of kanamycin sulfate (2,000 µg/ml Km; Bristol Laboratories, Syracuse, N.Y.) and nalidixic acid (5,000 µg/ml Na; Sterling-Winthrop Research Institute, Rensselaer, N.Y.) were prepared in sterile distilled water and 0.1 *N* NaOH, respectively, and sterilized through membrane filtration (0.22 µm; Millipore Filter Corp., Bedford, Mass.). Disc antibiograms of donor, recipient, and transcipient organisms were determined with a standardized technique[11]; broth dilution tests were performed as described previously[12]. The donor organisms tolerated greater than 100 µg/ml Km and were sensitive to Na; *E. coli* 1485-Na-R was inhibited by 3 µg/ml Km and tolerated greater than 100 µg/ml Na.

Transfer of aminoglycoside antibiotic resistance from the donor organisms to *E. coli* 1485-Na-R in vitro was accomplished by the technique of ANDERSON and LEWIS[13,14]. Samples (0.05 ml) from co-cultivated organisms ($1.5 \times 10^7$ donor and $1.5 \times 10^8$ recipient organisms/ml in a total of 20 ml nutrient broth at 0 time; incubated at 35 °C for 18 h) and control donor and recipient cultures were spread on MacConkey agar (Difco) plates containing 20 µg/ml Km (MAC-Km), 50 µg/ml Na (MAC-Na), 20 µg/ml Km + 50 µg/ml Na (MAC-Km-Na), or no drug (MAC). Plates were incubated at 35 °C for 24 h and examined for the presence of transcipients[15], which were subcultured to MacConkey agar, identified biochemically, and disc diffusion susceptibility tested.

For transfer of drug resistance in vivo, groups of 3 viable, 8-day-old, specific-pathogen-free chick embryos (Truslow Farms, Inc., Chestertown, Md.) each were inoculated into the allantoic cavity with 0.2 ml of the donor-recipient mixtures (the organisms were mixed in the same ratio as above immediately prior to inoculation), as well as organisms in isotonic saline, respectively. Control embryos received saline alone. Following incubation at 37 °C for 18 h, the chick embryos were candled, survivors were chilled for 1 h, and 0.05 ml aliquots of harvested allantoic fluid were spread on plain and selective MacConkey agar plates. Transcipients were processed as above.

In vitro transfer of aminoglycoside antibiotic resistance from *E. coli* 1513 (I) and *K. pneumoniae* isolate 829 (II) to *E. coli* 1485-Na-R (III) was readily achieved (Table I); resistance markers to Km and neomycin were transferred regularly, while resistance to streptomycin was transferred irregularly. Similar results were obtained when bacterial conjugation took place within the allantoic

[1] T. WATANABE, Bact. Rev. *27*, 87 (1963).
[2] N. D. REED, D. G. SIECKMANN and C. E. GEORGI, J. Bact. *100*, 22 (1969).
[3] E. H. THIELE, E. L. DULANEY, M. J. CAREY and D. HENDLIN, J. Bact. *95*, 1184 (1968).
[4] R. T. JONES and R. CURTISS III., J. Bact. *103*, 71 (1970).
[5] J. R. WALTON, Nature, Lond. *211*, 312 (1966).
[6] H. JAROLMEN and G. KEMP, J. Bact. *99*, 487 (1969).
[7] P. A. M. GUINEE, J. Bact. *102*, 291 (1970).
[8] A. I. BRAUDE and J. S. SIEMIENSKI, J. clin. Invest. *47*, 1763 (1968).
[9] H. W. SMITH, Lancet *1*, 1174 (1969).
[10] W. H. TRAUB, E. A. RAYMOND and J. LINEHAN, Appl. Microbiol. *20*, 303 (1970).
[11] A. W. BAUER, W. M. M. KIRBY, J. C. SHERRIS and M. TURCK, Am. J. clin. Path. *45*, 493 (1966).
[12] W. H. TRAUB, Appl. Microbiol. *20*, 98 (1970).
[13] E. S. ANDERSON and M. J. LEWIS, Nature, Lond. *208*, 843 (1965).
[14] M. J. LEWIS, Lancet *2*, 953 (1967).
[15] V. HINSHAW, J. PUNCH, M. J. ALLISON and H. P. DALTON, Appl. Microbiol. *17*, 214 (1969).