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## Effect of Hemolytic Streptococci on Tissue Cells.* (29154)

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The etiologic relationship of certain infections of the upper respiratory tract to group A hemolytic streptococci is well established and the further relationship of these infections to rheumatic fever and acute glomerulonephritis is highly probable, hence it seems important to learn more about host-parasite relations. Host-parasite relations at the cellular level are difficult to study in the patient and although tissue culture techniques at best are not a true counterpart of what happens in the patient, these techniques have some application in the study of these relationships. In this paper are reported experiments designed to study the spacial relationship and effect of group A streptococci on human tissue cells growing in tissue culture, and the effect of the tissue cells on the bacteria.

*Methods and materials. Tissue cells.*† Human tonsils were the source of lymphocytes. The tonsils were cut into fine pieces within 60 minutes of removal. The fibrous tissue was separated and the lymphoid tissue minced as fine as possible with scissors. The mince was filtered through sterile gauze. The filtrate, which contained the lymphocytes suspended in about 20 ml of medium, was composed of equal parts of mixture 199 and Eagle's basal medium containing 5% human serum, 40 μg of neomycin/ml, 1000 units of mycostatin/ml, and 100 units each of penicillin and streptomycin/ml. The cells were separated by centrifuging at 2500 r.p.m. for 15 minutes, resuspended in 3 ml of the above medium, then incubated for 2 hours at 37°C. Two-tenths to 0.3 ml of packed lymphocytes were the usual yield from each pair of tonsils. The lymphocytes were usually bacteriologically sterile after this treatment. They then were washed 3 times in the culture medium, taken up in 3 ml of medium without antibiotics and distributed in tubes, 1 ml per tube.

Cell preparations of human tonsil,‡ human liver and human monocytes were made by sub-culturing cells in antibiotic-free medium in Leighton tubes containing coverslips of No. 1 thickness. Each tube contained 0.8 ml

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† Cell lines used in these experiments and growth medium for each type of cell were human tonsil epithelial-like cells and mixture 199 + 10% calf serum; human liver cells and Eagle's Basal medium + 10 % calf serum + 0.01 mg L-glutamine/ml; human monocytes and Eagle's Basal medium + 10% calf serum + 0.01 mg L-glutamine/ml; monkey heart and minimal essential medium + 10% calf serum; monkey kidney and mixture 199 + 10% calf serum; human heart (Girardi) and minimal essential medium + 10% human serum + 0.01 mg L-glutamine/ml; human lymphocytes and equal parts of mixture 199 and Eagle's Basal medium + 5% human serum. Unless antibiotic-free medium was required, 1 mg each of penicillin and streptomycin was added to each 10 ml of medium.

‡ The tonsil cells were from a line of human tonsil epithelial-like cells obtained from Dr. Alfred S. Evans, Dept. of Preventive Med., Univ. of Wisconsin School of Med., Madison.

of medium. When a good growth of cells was present on the coverslip, usually within 2-3 days, the tubes were inoculated with streptococci and, at appropriate intervals, coverslips were removed from the Leighton tubes, laid face down on a glass slide on a drop or two of medium from the same tube, and rimmed with nail polish to make a tight seal. The "chambers" formed in this way were observed under a phase-contrast microscope.

Human heart, monkey kidney and monkey heart cell lines were grown in plastic dishes§ containing 4.0 ml of the appropriate growth medium. A round hole 1 cm in diameter was cut in each dish and covered with a glass coverslip 0.1 mm in thickness to make a seal, to facilitate observation and keep working distances for the oil immersion objective within the workable range. Observation of the cell lines growing in these dishes was enhanced by use of an inverted objective, phase-contrast microscope.

The cells tolerated a pH in the alkaline range very poorly; therefore the human heart and monkey kidney and heart cells which were growing in petri dishes were gassed with 5% $CO_2$ in air and incubated in a 5% $CO_2$ in air environment.

*Bacteria.* Liver cells and monocytes were inoculated with 2 strains of group A streptococci, a type 12 and a non-typable strain. Tonsil cells were inoculated with Group A, types 12, 6, and a non-typable strain. Lymphocytes were inoculated with Group A, type 12, streptococci. The experiments in which human heart and monkey kidney and heart cells were used were designed to test the effect of a large number of different strains of streptococci on these 3 cell lines and to determine whether differences in cell line susceptibility to damage by group A streptococci existed. The strains of group A hemolytic streptococci belonged to serological types 1, 3, 4, 5, 6, 7, 8, 9, 11, 12 (4 strains), 13, 14, 15, 17, 18, 19, 22, 23, 25, 26, 28, 29, 30, 31, 32, 33, 34, 36, 39, 41, 42, 43, 46, 47, and one non-typable strain. Strains of group C and group G were also used.

In the earlier experiments with liver and tonsil cells and monocytes, each loopful of inoculum contained approximately 0.02 ml with 2,400,000 chains of streptococci. The inoculum of streptococci for the monkey heart and kidney and human heart cells ranged from 48 to 4590 chains at first. However, since in these experiments in which these tissue cells were growing in 4.0 ml of medium in Cooper Falcon dishes, no significant differences were seen in the end result with smaller or larger numbers of streptococci in the inoculum, a single loopful 2 mm in diameter of a $10^4$ or a $10^6$ dilution of streptococci growing in Todd-Hewitt Broth was inoculated.

In the experiments with monkey heart, monkey kidney and human heart cells, growing in Cooper-Falcon plastic dishes, each dish received an average of 186,564 cells, the range being from 25,000 to 1,055,000 tissue cells. The cells were incubated 24 hours in 4.0 ml of medium and then examined. If the cells appeared healthy and were multiplying, the top of the dish was removed, the medium changed, inoculated with streptococci, gassed with 5% $CO_2$ in air, the top sealed and then incubated at 35°C in a 5% $CO_2$ in air atmosphere. Throughout these experiments, for each dish of cells inoculated with streptococci or fed medium which contained streptococcal products, a control dish of cells without streptococci or streptococcal products was always set up and incubated under identical conditions in order to compare "treated" with "untreated" cells.

*Criteria for cytopathogenic effect (CPE) on tissue cells.* These were similar to those outlined by Paul(1) for viruses and various cell lines, but also included other changes as follows: *Nuclear membrane.* Discoloration or thickening of wall. *Cytoplasmic granulation.* An early indication of alteration in the cell was the appearance of increased numbers of coarse granules in the cytoplasm. Nucleus remained unaltered longer than the rest of the cell. *Adherence to the glass or plastic.* Dead cells would float free in the medium and finally disintegrate. *Cell detail and out-*

§ Cooper tissue culture dish, 60 x 15 $mm^3$, by Falcon Plastics.

*line.* As destruction progressed the cell detail and cell outline became less distinct, with occasionally gradual swelling of the entire cell. *Cytoplasmic blebs.* Out-pouching of clear blebs of cytoplasm was seen early in the course of cell damage. *Vacuoles.* Vacuolation was seen in the cytoplasm as destruction progressed. Complete destruction was characterized by complete loss of cell outline and appearance of large clumps of cellular debris often mixed with large concentrations of streptococci. Loss of cell content occurred by rupture of the cell wall.

*Test for viability.* One ml of trypan blue 1% in normal saline was added to 5 ml of cell suspension. The cells were mixed with a pipette and counted. Dead cells turn blue, viable cells remain colorless and translucent.

*Effect of streptococcal growth products.* To test the effect of streptococcal and tissue cell growth products on tissue cells, 200 units of penicillin and 200 $\mu$g of streptomycin were added to each dish after 17-24 hours growth and allowed to incubate 2 hours. This invariably killed the streptococci. The medium was removed and centrifuged to sediment the streptococcal chains. This supernate was added to fresh medium in dilutions of 1 part used to 4 parts fresh medium and then substituted for the medium of healthy, growing cells. These dishes were observed for 48 hours for evidence of cell damage. Uncentrifuged as well as centrifuged streptococcal and cellular products were tested in this way.

*Effect of streptolysin O, streptolysin S and hyaluronidase.* The effect of streptolysin O, streptococcal hyaluronidase and Todd Hewitt Broth in which group A type 12 had been incubated was determined on human heart and monkey kidney and monkey heart cells. The streptolysin O from a strain of group A type 3, a strong streptolysin O producer, was used in both the unreduced and the reduced state. Streptolysin O was reduced by addition of cysteine, 1.5 mg/ml of broth. Streptococcal hyaluronidase was that produced by a strain of group A type 4. Each of these products was diluted $10^1$, $10^2$, $10^3$, and $10^4$, mixed with growth medium in a ratio of 1:5, added to the dishes of growing healthy cells and incubated 24 hours in 5% $CO_2$ in air. The effect of streptolysin S was tested by incubating human heart cells for 24 hours in medium containing 1400 hemolytic units of streptolysin S per ml of medium.‖

*Results.* The inoculum of streptococci in the earlier experiments was very large. One loopful when incubated on a plate containing 5% sheep's blood in agar produced at least 2,400,000 (colonies). Results obtained with these large numbers of bacteria were quite consistent. Each of the strains of streptococci multiplied and was lethal for each of the 4 types of tissue cells (human tonsil, human monocyte, human liver, and human lymphocyte), complete destruction taking place within 2 days maximum, and usually in much less time. The non-typable strain seemed to be more destructive than the type 12 or 6, complete lysis of the tissue cells occurring within 24 hours.

Inoculation of tonsil cells growing on slides in Leighton tubes or small bottles with streptococci in smaller numbers gave results which were not predictable, at least not within the range of numbers of infecting streptococci used, which was from 179 to 14,545 chains. Within this range of streptococci in the inoculum, streptococci did not always survive. When they did survive and multiply, the tonsil cells in some instances were killed but in some instances survival of both streptococci and cells resulted in a kind of symbiotic relationship. This did not seem to be a strain characteristic of the streptococci because both kinds of relationships were seen with the same strain of streptococcus. Suggestive evidence that the tonsil cells had an inhibitory effect on the multiplication of streptococci was noted in experiments comparing growth of group A, type 6, streptococci in the presence and in the absence of tonsil cells. When growth medium was inoculated and allowed to incubate 18 hours, colonies of streptococci were too numerous to count when 0.1 ml of $10^3$ to $40^5$ dilutions of the growth medium were streaked on blood agar plates and incubated for 18 hours. When tissue cells were present, the number of colonies was signifi-

‖ Streptolysin S was obtained from Dr. Alan W. Bernheimer, Dept. Microbiology, New York Univ. College of Med.

TABLE I. Growth of Group A Type 6 Beta Hemolytic Streptococci with and without Human Tonsil Cells.

| Growth medium | Colony count* Before incubation† | Colony count* After incubation† | Inocula streaked on blood agar plates to count colonies† |
|---|---|---|---|
| Mixture 199 | 182-349 | Too numerous to count | |
| " " + 12% calf serum | 32-427 | *Idem* | .1 ml of $10^3$ to $40^5$ dilutions |
| Mixture 199 + 12% calf serum + human tonsil cells | 12-458 | 458-6860 | |

* Colony counts are the range of counts from several experiments.
† Incubation at 37°C for 24 hr.

cantly fewer, indicating some inhibition of streptococcal growth (Table I). It is possible that the amount of medium available in the Leighton tubes (0.8 ml), was not enough to support optimal growth of both streptococci and tissue cells. In later experiments with 4.0 ml of medium, nutritional factors of used medium were still adequate because medium in which streptococci and cells had grown simultaneously would still support cell growth and bacterial growth. The possible inhibitory effect of tissue cells on streptococcal growth will be studied further.

Tonsil cells seemed to "hold" streptococci in close proximity to cell wall by means of multiple, very thin, thread-like tentacles considered to be evidence of cell damage, which radiated from all parts of the cell. Within 3-4 hours after inoculation and occasionally within an hour, especially with a heavy inoculum, chains of streptococci could be seen lining the small crevices and indentations between the tonsil cells and thread-like tentacles. After longer periods of incubation, the bacteria occasionally formed "blankets" around the cells. Only on 2 occasions (with type 6) among the many hours of observation, were streptococci seen intracellular (tonsil cell) following a large inoculum. It was not necessary for streptococci to be in contact with or even in close proximity to tonsil cells for cell destruction to occur.

Lymphocytes survived in tissue culture medium for as long as 12 days. After this time most of the lymphocytes appeared dead, smaller lymphocytes surviving the longest, larger lymphocytes disintegrating earlier. Lymphocytes infected with a heavy inoculum of type 12 streptococci were killed in about 4 hours. There did not seem to be much change in the lymphocytes before 3 hours, but thereafter destruction was rapid, and was complete after 22 hours. Intracellular streptococci were not observed, which is understandable because lymphocytes are not phagocytic.

When monkey kidney and human heart cells were inoculated with 37 typable strains of group A streptococci, including 4 different strains of type 12, a non-typable strain of group A, a group C and a group G, both cell lines were susceptible to damage by most strains of streptococci under the conditions of these experiments. However, human heart cells were vulnerable to more strains of streptococci than monkey kidney cells. Data in Table II show that human heart cells were completely destroyed alone on 20 occasions, both human heart and monkey kidney 11 times, monkey kidney alone twice and in 7 experiments neither cell line was affected. Strains of streptococci used in the above experiments are also shown in Table II.

Cells survived and multiplied in a pH range from 6.8 to 7.8 but at pH 8.0 cells died. Even when streptococci had previously been growing in the "used" medium, the cells remained healthy, showing that streptococcal products were not harmful to the cells under these conditions.

Streptolysin O (reduced or unreduced), streptococcal hyaluronidase, streptolysin S, and Todd-Hewitt Broth in which group A type 12 had grown, had no observable effect

TABLE II. Effect of 40 Different Strains of Hemolytic Streptococci on Human Heart (HH) and Monkey Kidney (MK) Cells After 24 Hours Incubation.

| No CPE* on either HH or MK | Effect on tissue cells | | |
|---|---|---|---|
| | Complete destruction of both HH and MK | Complete destruction of HH | Complete destruction of MK |
| 7 | 11 | 20 | 2 |
| | Strains of Group A streptococci causing the above effects | | |
| 7, 15, 19, 32, 36, 39, 46 | 5, 13, 14, 17, 18, 25, 28, 29, 47, (Group C and Group G) | NT, 1, 3, 4, 6, 8, 9, 11, 12 (4 different strains), 22, 23, 26, 30, 31, 41, 42, 43 | 33, 34 |

* CPE = Cytopathogenic effect.

on human heart, monkey kidney or monkey heart cells during the observation period of 24 hours.

*Discussion.* These experiments show that many types of hemolytic streptococci belonging to serological group A and strains of group C and group G are able to kill human heart, monkey heart, and monkey kidney cells. The surprising thing is that none of the growth products from any of these strains had any observable effect on these same cell lines. For cell damage to occur, living streptococci must be present in the tissue culture medium.

That lymphocytes and human heart cells were highly susceptible to damage by streptococci and that human heart cells were more suscepible to damage by more streptococcal strains than monkey kidney or monkey heart cells, suggests that there are inherent differences between cells of different species in their resistance to damage by the hemolytic streptococcus.

Large inocula of group A streptococci, type 12 and 6 and a non-typable strain, were highly lethal for liver cells, tonsil epithelial-like cells, monocytes and lymphocytes from human sources. It would appear that large inocula of group A hemolytic streptococci consistently overwhelm these tissue cells. With smaller inoculating doses of bacteria, survival and apparent symbiosis of streptococci and tissue cells occur in some tubes, but result in complete destruction of tissue cells in others.

As our results are not strictly comparable with the results of others using different cell lines or tissues, it is not surprising that they are at variance with those of Shepard(2) who showed that group A streptococci took only 3 hours to fill HeLa cells growing in tissue culture. However, HeLa cells are phagocytic. Holland and Pickett's(3) experiments were concerned mainly with Brucellae, but they presented evidence that group A streptococci grew intracellularly in chick embryo fibroblasts. It seems evident from the work of Goodpasture and Anderson(4) that streptococcus hemolyticus grows by preference on the surface of the ulcerated chick and embryonic membrane and on necrotic tissue. There is no evidence from their work that streptococci proliferated within cells of the exudates or in the tissues of the host, although they may remain viable in the body of the living embryo. There was no evidence of direct invasion of the embryonic tissues in sections of infected membranes.

In pharyngitis and tonsillitis due to the group A hemolytic streptococcus there is little information on the spacial relationship between streptococci and tissue cells or on pathological effects of the bacteria on tissue cells; likewise, in the so-called carrier state. Previous studies of the bacterial flora of tonsils have dealt primarily with cultural characteristics of bacteria obtained from the surface or crypts. Bacteria of a great variety were described lying in the intercellular spaces but exact location was not specified (5). Parkinson(6) stated that in cases of follicular tonsillitis, bacteria of unusual virulence gain entrance to the crypts, the toxins from the bacteria cause swelling of the epithelial cells of the cryptal lining, then swelling of the cells is followed by disintegration and necrosis which result in ulceration of parts of the epithelial lining. It is tempting

to think of the events occurring in tissue culture as counterparts of what might happen in human infections but it is obvious that such is not the case; more study is necessary.

*Summary.* Human tonsil and liver cells and human monocytes and lymphocytes were inoculated with hemolytic streptococci belonging to serological groups A, C, and G. A large inoculum of bacteria invariably resulted in complete destruction of the above tissue cells. Smaller inocula of bacteria gave variable results ranging from complete destruction of tissue cells to complete destruction of the streptococci and in some cases apparently a symbiotic relationship in which both tissue cells and bacteria survived. Human heart cells were susceptible to damage by a larger number of strains of streptococci than were monkey kidney cells. Used medium in which streptococci and tissue cells had grown had no observable effect on human heart, human tonsil, monkey kidney, or monkey heart cells. Streptolysin O (reduced and non-reduced), streptolysin S, streptococcal hyaluronidase, and Todd-Hewitt broth in which streptococci had grown had no observable effect on the above tissue cells.

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## A Simple Method for Obtaining Blood from Ducks. (29155)

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Special technics have been developed for obtaining blood from guinea pigs(1), mice (2), rats(3), rabbits(4), chickens(5), etc. Recently we were faced with the problem of obtaining repeated blood samples from Pekin ducks. Initially, the usual method for obtaining blood from fowls was used, *i.e.,* cutting down to the brachial vein in the wing. After this was repeated several times, scar tissue formed which made the procedure difficult. This report concerns a method for obtaining blood from ducks which requires no surgery, is time saving and reliable.

*Method.* The duck is laid on its side on a narrow laboratory table, such as is used for animal surgery, the hindquarters covered with a towel, the neck and head held down by a strap, and the legs extended away from the person holding them.

The vein is located along the medial aspect of the shank of the duck's leg. Usually a pulse can be felt and the outline of the vein can be observed in the area of the pulsation. Washing the hard scaly skin over the area with saline sometimes helps to make the vein more visible when it is difficult to see. Fig. 1-a shows the location of the vein as it circles around the hallux (which corresponds to the spur of the rooster), and goes up the shank of the leg until is disappears in the flesh and feathers at the hock joint.

A 20 gauge needle attached to a 10 ml syringe is inserted into the vein in the region of the hallux. Care should be taken not to get the needle too high or it might injure the hock joint. If withdrawn fairly slowly so as not to collapse the vein, 30 to 40 ml of blood can be withdrawn without difficulty. Vacuum blood collecting tubes should not be used for drawing the blood as they cause the vein to collapse.

Fig. 1-b shows the location of a vein in the lateral aspect of the leg. This vein is just as satisfactory as the other, but it is a little