phosphorylated compounds as intermediates whether they are hydrolyzed and the glycerol reutilized after phosphorylation or whether they are directly phosphorylated to lysophosphatidic acids.

1. Clement, G., Paris, R., C. R. Acad. Sci., Paris, 1964, v958, 6006.
2. Clark, B., Hubscher, G., Biochim. Biophys. Acta, 1961, v46, 479.
3. Di Costanzo, G., Clement, J., Bull. Soc. Chim. Biol., 1963, v45, 137.
4. Abramson, D., Blecher, M., J. Lipid Res., 1964, v5, 628.
5. Pieringer, R. A., Hokin, L. E., J. Biol. Chem., 1962, v237, 653.
6. Mattson, F. H., Volpenhein, R. A., ibid., 1964, v239, 2772.
7. Weiss, S. B., Kennedy, E. P., J. Am. Chem. Soc., 1956, v78, 3550.
8. Pieringer, R. A., Hokin, L. E., J. Biol. Chem., 1962, v237, 659.
9. Ailhaud, S. D., Lazdunski, M., Desnuelle, P., Biochim. Biophys. Acta, 1964, v84, 643.

Received March 10, 1965. P.S.E.B.M., 1965, v119.

Tissue Culture of Tonsillar Lymphocytes.* (30247)

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It is well established that lymphoid tissue is involved in the immunologic reactions of experimental animals. In cases of severe allergic systemic reactions in man, the regional lymph nodes frequently become enlarged. As part of a study of the allergic reaction in human subjects, we used tonsils from patients in apparent good health. These tonsils were considered a prototype of "normal" lymphatic tissue. Cultures of these tonsils yielded a variety of cells, consisting predominantly of small lymphocytes with smaller numbers of plasmacytes, large lymphocytes and other cells which were similar to those described in histologic sections of tonsils(1). We have found that the growth of the tonsillar lymphocytes in tissue culture was considerably improved by the presence of human gamma globulin.

Materials and methods. The basic medium used was Eagle's minimum essential medium (MEM, Microbiological Associates) containing fetal calf serum at a concentration of 15%. Penicillin-G (100 units/ml) and streptomycin sulfate (50 μg/ml) were added. The medium was sterilized by passage through a 0.54 μ Millipore® filter. Trypsin (1:250, Difco Laboratories) was used at a concentration of 0.5% (w/v) in Hanks' balanced salt solution (BSS, Microbiological Associates). The proteins (Pentex Corp.) were crystalline preparations of human serum albumin (HSA), bovine serum albumin (BSA), human gamma globulin (HGG) and egg albumin (OA).

Tonsils were obtained from male and female donors between the ages of 7½ months and 14 years, most being between 2 and 6 years. A portion of tonsil was finely minced with sterile scissors, washed 3 times with BSS, centrifuged and resuspended in 0.05% trypsin solution. A fairly uniform suspension was obtained after trypsinization at room temperature (23°-26°C), using a magnetic stirrer for about 15 to 20 minutes. The suspension was allowed to settle for about one minute and the supernatant was withdrawn and centrifuged at 1500 rpm in a refrigerated centrifuge. The cells were washed twice with basic medium by resuspension and centrifugation and finally dispersed in 5 to 10 ml of basic medium. The precise volume was determined by the number of culture bottles to be set up. Each bottle, containing 9.5 ml of medium and 0.5 ml of the cell suspension, was incubated at 37°C in an atmosphere of air and 5% CO_2. The control samples were cultured in MEM with 15% fetal calf serum

* This work was supported by USPHS Grant AI 05386.

(basic medium) and the experimental samples in basic medium containing one of the crystalline proteins (5 mg/ml). The control and experimental bottles were prepared in duplicate.

Twenty-four hours after inoculation, the supernatant fluid was decanted and replaced by an equal volume of the original medium. The cultures were observed microscopically each day through the glass wall to which the cells were attached. After the first change of medium, it was rarely necessary to make another change as judged by the color of the medium (pH) and the condition of the cells. After 3 to 9 days of incubation, the cultures were carefully scraped from the wall with a rubber policeman. The cell suspensions were centrifuged at 800 rpm in a refrigerated centrifuge, resuspended in 5.0 ml of medium and counted in a hemocytometer, after dilution with an equal volume of a 0.1% solution of crystal violet in 1% acetic acid. The cells contained in 9 hemocytometer squares were counted and, if fewer than 100 cells were present, further counts were made on other aliquots of the cell suspension. The remainder of the suspension was centrifuged at 800 rpm and the pellet was used for the preparation of smears which were fixed with ethanol acetic acid (3:1 v/v) and stained with Wright's stain.

Plasma clot cultures were performed as described by Paul(2).

Results. The cultures consisted of small lymphocytes (80%), large lymphocytes (10%), plasma and other cells (10%). This composition did not vary significantly during incubation times up to 9 days. The total cell count increased for the first 2 to 3 days, then remained constant up to 9 days of incubation (Fig. 1). The cultures containing HGG (B',C') grew faster than the control cultures (B,C) for the first 2 days, after which the growth curves remained roughly parallel.

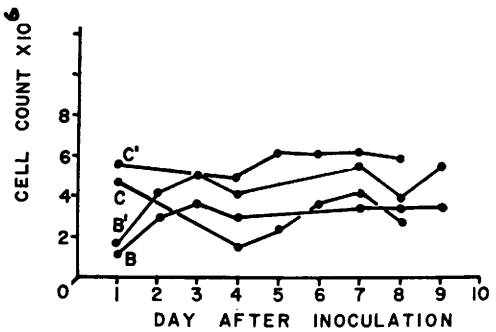

FIG. 1. Changes in numbers of tonsil lymphocytes per bottle with time. B and C represent control bottles containing only basic medium. B' and C' represent bottles containing HGG (5 mg/ml) in basic medium. Each letter corresponds to a separate tonsil.

TABLE I. Action of Purified Protein Antigens on Growth of Tonsillar Tissue Cultures.

Protein 5 mg/ml	Experiments conducted	Avg ratio of test/control
HSA	6	1.11 ± .10*
BSA	6	.82 ± .012*
HGG	9	2.31 ± .34*
OA	5	1.20 ± .17*

* Standard error of mean.

The good agreement between the number of cells in duplicate cultures (less than 10% difference) supported the conclusion that differences between the number of cells in the control and experimental cultures were real and due to a specific alteration of the environment. We, therefore, "normalized" the data by determining the ratio of the cell count in the experimental group to that in the controls. Fig. 2 shows the ratios for tonsillar lymphocytes cultured in the presence of HSA, BSA, HGG and OA. Table I summarizes the mean ratios obtained in all the experiments. It can be seen that addition of HSA, BSA and OA resulted in ratios which were not significantly different from 1.0. On the other hand, the addition of HGG resulted in a mean ratio of 2.3 with values as high as 4.0 (Fig. 2). Each of 13 tonsils was used to prepare 3 cultures: one in basic medium, one with HGG and one with another protein. In all cases, only the cultures with HGG showed increased proliferation. Table II shows that one to two mg of HGG per ml of medium were sufficient to cause nearly maximum increase in cellular growth in two typical cultures of 3- and 5-day duration.

All results reported above were obtained using tonsil tissue as a starting material. To find out whether the observed increase in cell ratio resulted from an increase in rate of cell multiplication or from a decrease in rate

of cell disappearance, we grew tonsil fragments in plasma clot culture(2) for 6 days, separated the cells from the fragments by mild trypsinization and washed them. 41 $\times$ 10^4 cells were inoculated into each of 4 bottles, 2 of which contained basic medium and 2 basic medium plus HGG (5 mg/ml). After 3 days, the control bottles contained 33 $\times$ 10^4 and 36 $\times$ 10^4 cells and the experimental bottles contained 80 $\times$ 10^4 and 85 $\times$ 10^4 cells.

Discussion. During the early stages of this investigation, attempts were made to count the number of lymphocytes and other cells in the initial suspension. The amount of debris and red cells was so great as to make it exceedingly difficult, if not impossible, to make any meaningful count. In view of Rebuck's(3) finding that lymphocytes adhere to glass, we allowed the cell culture to incubate for 24 hours and poured off the supernatant suspension containing the debris. We realize that some intact cells may have been discarded with this procedure, but we have focused our attention on the cells (mostly lymphocytes) which remained attached to the glass wall. These cells resemble the lymphoid cells of tonsils, lymph nodes and spleen(1). Since their morphologic appearance and the fraction of small lymphocytes relative to the total number of cells did not change during the period of observation, only the total number of cells could be used to determine the effect of the added proteins on the cultures.

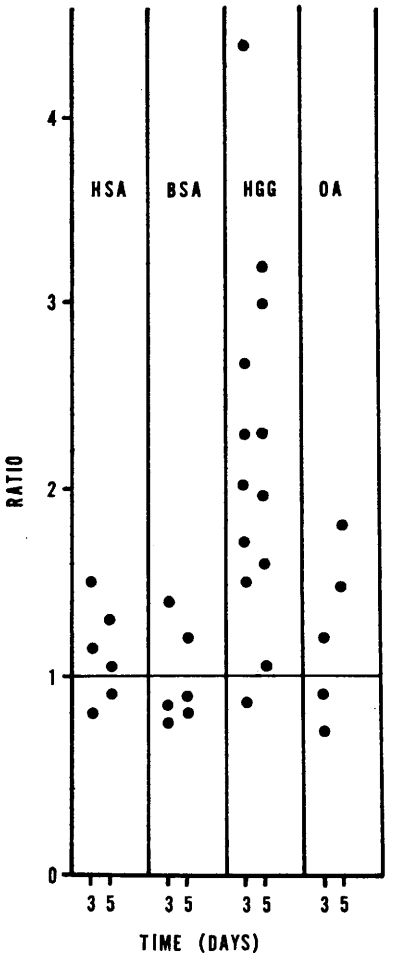

FIG. 2. Effect of various proteins on ratio (number of cells in experimental bottles: number in control bottles) after 3 and 5 days of incubation. Each point corresponds to a different tonsil. Abbreviations explained under *Materials and methods.*

It is of interest to compare the behavior of tonsillar and circulating lymphocytes in tissue culture. According to Nowell(4), Robbins(5) and to our own observations, cultures of circulating lymphocytes contain a significant fraction of large lymphocytes which increases greatly in the presence of various reagents, *i.e.*, PHA. We have not seen this increase in any culture of tonsillar lymphocytes. Indeed, one of the outstanding characteristics of a colony of tonsillar tissue is the constant percentage of small lymphocytes (78-83%) in the culture regardless of method of treatment. In some preliminary studies of the effect of HGG on circulating lymphocytes, we have not observed either an increase in number of cells or a change in the percentage of large forms. On the other hand, we have found that phytohemagglutinin (PHA), which causes an increase in the percentage of large forms in cultures of circulating lymphocytes(4), did not do so in cultures of tonsillar lymphocytes, the growth of which appeared to have been inhibited by it.

In considering the mode of action of HGG on tonsillar lymphocytes in culture, several possibilities present themselves. First, HGG may act by increasing the oncotic pressure of the medium. This possibility may be ex-

TABLE II. Effect of Concentration of HGG.

No. of days	Control*	Avg ratios of test/control†					
		+.5 mg/ml‡	+1 mg/ml‡	+2 mg/ml‡	+3 mg/ml‡	+4 mg/ml‡	+5 mg/ml‡
3	24.5×10^4 cells/ml	1.14	1.46	1.53	1.21	1.56	1.63
5	42.6×10^4 cells/ml	1.06	1.28	1.74	1.63	—	1.9

* Number of cells in control bottles.
† Ratio equals number of cells per ml in experimental bottle: number in control.
‡ Concentration of HGG in medium.

cluded since other proteins, added in comparable amounts, did not have the same effect. The absence of an optimal concentration of HGG and the observation that with increasing concentration of HGG the degree of stimulation did not change (Table II) also eliminates the osmotic effect as a primary cause. Second, HGG may increase the adhesion of the lymphocytes to the walls in a manner similar to that described by Fisher, Puck and Sato for fetuin(6). Third, HGG may stimulate the growth or multiplication of tonsillar lymphocytes in a manner similar to that of PHA on circulating lymphocytes(4). Both increased adhesion and multiplication may occur together since Puck(6) and Kimura (7) have show that, in general, the presence of a solid surface is necessary for the growth of cell cultures. Finally, HGG, or a component of HGG, may have a stimulatory effect specific for tonsillar and/or other lymphoid cells, but not for circulating lymphocytes. This is not the same as the third alternative since the action of PHA or other materials on circulating lymphocytes has been shown to be related to their antigenic properties(8). It is difficult to assume a similar "antigen-like" action by a homologous globulin. On the basis of present knowledge, it is reasonable to asume that the fourth mechanism plays a major role and that the second and third ones, operating together, also play a part in the growth of tonsillar lymphocytes in tissue culture.

Summary. Tonsils have been grown in tissue culture. The culture consists of 80% small lymphocytes, 10% large lymphocytes and 10% a mixture of other cells. It has been found that human gamma globulin stimulates the growth of these cultures whereas human serum albumin, bovine serum albumin and ovalbumin have no effect.

1. Maximow, A. A., Bloom, W., A Textbook of Histology, Saunders, Philadelphia, 1957.
2. Paul, J., Cell and Tissue Culture, 2nd Ed., Livingston Ltd., Edinburgh, 1960.
3. Rebuck, J. W., Petz, A. J., Riddle, J. M., Priest, R. J., Lo Grippo, G. A., Human Leucocytic Functions in the Tissues in Ciba Foundation Study Group 10, Little, Brown, Boston, 1961. See particularly the discussion on pp. 27ff.
4. Nowell, P. C., Exp. Cell. Res., 1960, v19, 267.
5. Robbins, J. H., Science, 1964, v146, 1648.
6. Fisher, H. W., Puck, T. T., Sato, G., Proc. Nat. Acad. Sci. (U.S.), 1958, v44, 4.
7. Kimura, R., Tissue Culture, Munksgaard, Copenhagen, 1953.
8. Ling, N. R., Husband, E. M., Lancet, 1964, 363.

Received March 15, 1965. P.S.E.B.M., 1965, v119.