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Transformation of Lymphocytes by Phytohemagglutinin in Leprosy Sera[1, 2, 3]

Pui Ching Wong, C. H. Chan-Teoh, S. Wu and F. H. Kendall[4]

It is generally held that some of the features of lepromatous leprosy are due to an anergy of the immunologic response and evidence is accumulating that this is related to defects in the small lymphocytes. Rodriguez Paradisi *et al.* ([16]) and Dierks and Shepard ([6]) have reported that lymphocytes from lepromatous patients do not undergo transformation as freely as those from normal persons when phytohemagglutinin (PHA) is added to them in tissue culture. Similar results were obtained by Bullock and Fasal ([3]) who showed that the addition of PHA apparently impaired the rate of DNA synthesis in leucocytes cultured from patients with leprosy. Merklen ([12]), however, reported that lymphocytes from tuberculoid leprosy did not undergo transformation whether they were stimulated by murine leproma or PHA but lymphocytes from lepromatous leprosy transformed under the same stimuli. Because of these different findings and the importance of their implication in the understanding of leprosy, it seemed desirable to examine these observations by a different procedure. In this work the liquid scintillation-counting technic was used to measure the effect of PHA on the incorporation of radioactive thymidine and uridine by lymphocytes taken from cases of leprosy

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[2] From Department of Microbiology and Radioisotope Unit, University of Hong Kong, Hong Kong.

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[4] P. C. Wong, M.D., Ph.D., M.R.C. Path., Associate Professor, Department of Bacteriology, University of Alberta, Canada; C. H. Chan-Teoh, M.D., Ph.D., Senior Lecturer, Department of Microbiology, University of Hong Kong; S. Wu, B.A., Research Assistant, Radioisotope Unit; F. H. Kendall, B.Sc., Ph.D., F.R.I.C., Director and Radiation Protection Officer, Radioisotope Unit, University of Hong Kong.

MATERIALS AND METHODS

Lymphocytes were obtained from the venous blood of three groups of Chinese people, i.e., from normal persons and from those with untreated lepromatous or tuberculoid leprosy at the time of diagnosis in Sai Ying Pun Clinic. The whole of the experiments described were carried out in duplicate although not at the same time.

Preparation of lymphocyte cultures. Each experiment began with the early morning collection of 13.5 ml. of blood into a syringe containing 1.5 ml. of 3.5 per cent sodium citrate solution from each of four individuals of one group.

This blood was pooled into a silicon-oil-treated test tube along with 1.5 ml. of 6 per cent dextran in isotonic saline and the red cells were allowed to sediment at room temperature for at least two hours. The supernatant fluid of leucocyte-rich plasma was withdrawn and run through a six-inch column of glass beads. The neutrophils adhered to the glass and so were removed and relatively pure lymphocyte suspensions were obtained ([15]). The lymphocyte content of the effluent plasma was checked with Giemsa-stained smears, the quantity was measured and the cells counted. The lymphocyte-containing plasma was centrifuged at 2,000 rpm for 10 minutes in silicon-oil-treated test tubes to obtain a concentrated deposit of lymphocytes. The concentrated cells in the sediment were counted and the concentration was adjusted by adding tissue culture medium to the sediment so that each 0.1 ml. of cell suspension contained 2 x 10^6 lymphocytes. Each 0.1 ml. sample was delivered into a tissue culture tube and diluted with 0.9 ml. of tissue culture medium and this constituted the final sample which was used for each measurement. The tissue culture

medium used throughout the experiments consisted of four parts of medium TC 199 and one part of calf serum (obtained from Flow Laboratories, Maryland, U.S.A.) with 100 units each of penicillin and streptomycin per milliliter.

To determine the effect of PHA on thymidine and uridine incorporation four sets of measurements were carried out as follows:

In the first set of tests, 2△5 μCi thymidine-6-T(n) (specific activity 5.0 curies/mM, Radiochemical Centre) in 1 ml. of tissue culture medium was added to each of seven samples of lymphocytes in culture tubes. In the second set, 0.1 ml. of PHA solution (Difco laboratories) was added to seven samples in addition to the same dose of radioactive thymidine. In the third set of tests 2.5 μCi/ml. of radioactive uridine-T (G) (specific activity 2.44 curies/mM, Radiochemical Centre) was added to each of the seven samples of lymphocytes in culture tubes. In the fourth set, 0.1 ml. of PHA was added to seven samples in addition to the same dose of radioactive uridine. The tubes were then incubated at 37°C and groups of four tubes (one from each set) were removed after 1 hr., 6 hrs., 12 hrs., 18 hrs., 24 hrs., 48 hrs., and 72 hrs.

Measurement of incorporation of tritiated thymidine and uridine. (Modified method of Kay and Korner ([9])). As soon as each group of four tubes was taken out of the incubator, the tubes were centrifuged for 10 minutes at 2,000 rpm and the supernatant culture medium decanted. Each sediment was washed 10 times with 5 ml. quantities of cold 0.15M NaC1 solution in

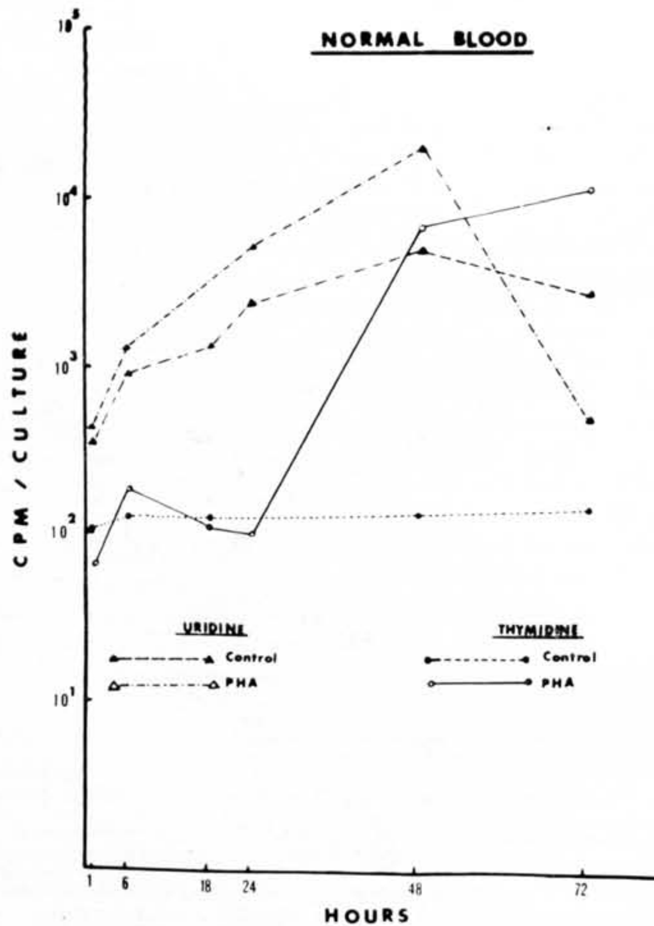

FIG. 1. Comparison of the responses of PHA-treated lymphocytes and untreated lymphocytes to tritiated uridine and thymidine in normal individuals.

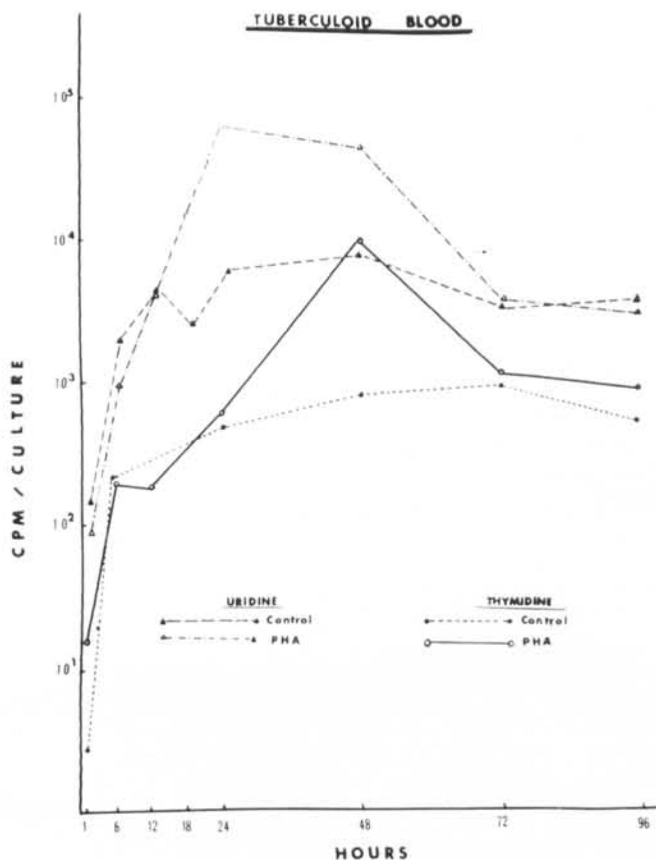

FIG. 2. Comparison of the responses of PHA-treated lymphocytes and untreated lymphocytes to tritiated uridine and thymidine in tuberculoid leprosy patients.

order to remove radioactive substances not incorporated in the cells; RNA and DNA were then precipitated by adding 5 ml. cold 0.5N $HC10_4$ solution to each tube and this brought about maximum precipitation in 10 minutes. These tubes were again centrifuged and the supernatant fluid decanted.

The precipitate in each tube was washed once with 5 ml. of 0.5N $HC10_4$ solution, then with 5 ml. of ethanol-ether (1:1 v/v), and finally with 3 ml. of ether. The precipitate was dried in the same tubes in an oven at 50-60°C and re-dissolved in 0.2 ml. of quartenary ammonium hydroxide (0.6N in toluene, supplied by Nuclear Chicago as NCS solubiliser). This solution was transferred to a scintillation counting vial containing 6. 8 ml. of a scintillation fluor comprising 2, 5-diphenyl-oxazole (3.5g/1) and 1,4-bis-(2-(5-phenyloxazole))-benzene (50 mg./1) in toluene. To obtain quantitative transfer, the tube was washed with six 0.5 ml. portions of fluor. Each sample was refrigerated at 4°C before counting in a Nuclear Chicago Liquid Scintillation Spectrometer. All samples were counted within two days after collection of the last samples.

RESULTS

All count rates were corrected for background counts and the averages from duplicate measurements were plotted against incubation times as shown in Figures 1-3.

Incorporation of radioactive thymidine into lymphocyte DNA. The experiments carried out on the lymphocytes from normal blood showed that without PHA, count rates remained low and fairly constant throughout the whole experiment, but when PHA was added, a marked increase was found in the samples incubated for 48 and 72 hours. Between 24 and 72 hours, the

count rate increased by about 100-fold over the unstimulated values. This is similar to the findings of other workers on PHA stimulation ([8]).

In experiments carried out with lymphocytes from tuberculoid leprosy the count rate remained fairly constant in the unstimulated lymphocytes after six hours as was the case with lymphocytes of normal individuals. The effects of PHA were less pronounced than with normal lymphocytes. Thus, at 48 hours only a 16-fold increase of count rate occurred (Fig. 2).

The experiments conducted with lymphocytes from lepromatous leprosy consistently showed no stimulating action by PHA on the lymphocytes, as illustrated in Figure 3.

Incorporation of radioactive uridine into lymphocyte RNA. Count rates were generally higher in the uridine samples than those with thymidine but all of these experiments showed a pronounced tendency for count rates to drop after long incubation periods. In the controls (Fig. 1) count rates increased steadily up to 48 hours and then fell away. With PHA stimulation, the count rates were three-to four-fold higher, up to the 48-hour reading.

The count rates from tuberculoid lymphocytes without stimulation increased rapidly up to 12 hours but then remained essentially constant (Fig. 2). In samples to which PHA was added the count rate was increased by as much as ten-fold at 24 hours, but the overall picture was not dissimilar to that for uridine samples of normal lymphocytes.

In samples of lepromatous lymphocytes, a well defined maximum count rate at six

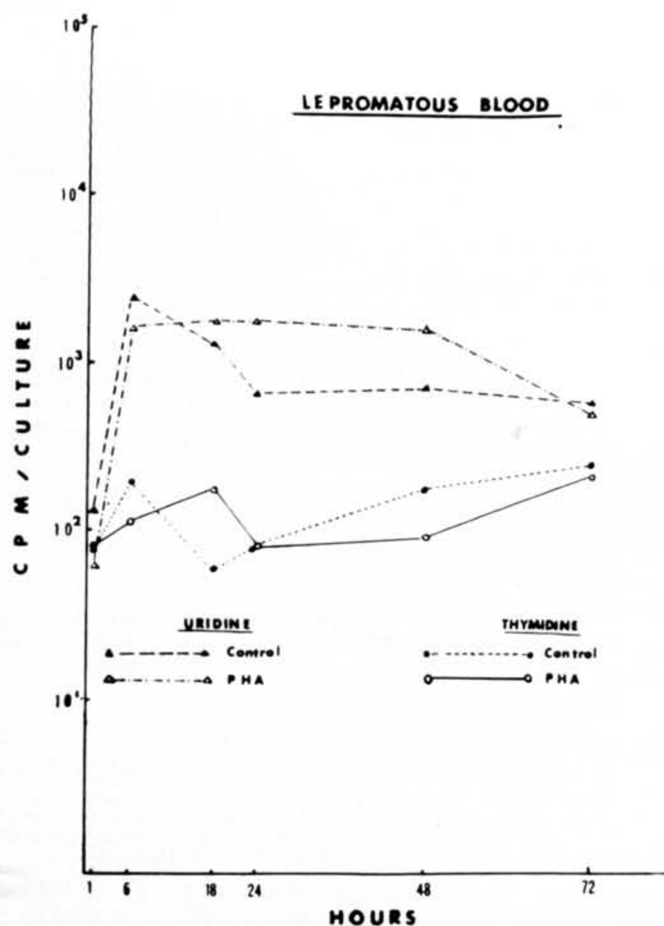

FIG. 3. Comparison of the responses of PHA-treated lymphocytes and untreated lymphocytes to tritiated uridine and thymidine in lepromatous leprosy patients.

hours was observed, followed by a steady fall-off. No stimulation by PHA was detected in these experiments (Fig. 3).

In general, the PHA stimulation of RNA and DNA syntheses as measured by the incorporation of tritiated uridine and thymidine respectively, was low in lymphocytes of tuberculoid cases and completely absent in lymphocytes from lepromatous cases.

The results illustrated in Figures 1, 2, and 3 show that for these lymphocytes, where incorporation of thymidine does occur, it is generally preceded by a considerable increase in uridine incorporation corresponding to RNA synthesis.

DISCUSSION

Immunity in mycobacterial infection is currently viewed as acquired cellular immunity as exemplified in tuberculosis ([5,-10]). This is mediated by macrophages and lymphocytes and can be studied by observing the transformation of circulating lymphocytes into "blast cells" by the stimulation due to PHA ([7, 13]). The transformation is associated with cell division and the small lymphocytes are described as immunologically competent ([14]). The mitosis and DNA synthesis of the transformed lymphocytes in cultures of human leucocytes have been studied using tritiated thymidine ([1]), and RNA synthesis using tritiated uridine ([4]). The transformation was preceded by an early rise in RNA synthesis ([11]).

The existence of two polar types of leprosy appears to be related to differences in immunity in the subject and the method of immune responses to different antigens has been applied to the study of leprosy ([2]). Rodriguez Paradisi *et al.* ([16]) reported that "blast cell" formation was diminished in lepromatous leprosy. Dierks and Shepard ([6]) showed that the response of lymphocytes from leprosy patients to PHA, PPD, and BCG stimulation was defective as revealed by the percentage of lymphocyte transformation, and this paralleled changes in the skin reactivity. The defect in response was greatest in their patients with active lepromatous leprosy but was also found to a lesser extent in tuberculoid leprosy and in lepromatous leprosy rendered inactive by therapy. Impaired skin reactivity to 2-4 dinitrochlorobenzene was noted in lepromatous leprosy by Waldorf *et al.* ([18]). They also reported impairment of lymphocyte transformation from lepromatous leprosy patients to streptolysin O antigen ([17]).

Bullock and Fasal ([3]) using tritiated thymidine incorporation as a measure of DNA synthesis found that there was impairment of synthesis in lepromatous leucocytes compared to normal leucocytes, as well as the synthesis of DNA after streptolysin O administration in autologous medium. The response of tuberculoid leucocytes to both PHA and streptolysin O was normal. They concluded that both humoral and cellular factors may be operated in depressing the immune response of leucocytes in cultures from patients with leprosy. The method used for measuring DNA synthesis by the uptake of radioactive thymidine was not given in detail.

We have not tested the effects of normal homologous serum on the behavior of lymphocytes from leprosy patients but our findings confirm those of Bullock and Fasal that when cells from lepromatous cases are exposed to PHA there is no increase in DNA synthesis as measured by tritiated thymidine. Depression of the immune response in lepromatous patients is reflected in impaired RNA and DNA synthesis by stimulated lymphocytes.

In our experiments the stimulating effect of PHA on thymidine incorporation into the lymphocytes of tuberculoid cases was considerably less than that in normal lymphocytes. Depression of DNA synthesis is also evident in this type of disease, but it is less severe than in lepromatous cases. The stimulating effect of PHA on uridine incorporation into lymphocytes from cases of tuberculoid leprosy was of approximately the same order as that in normal lymphocytes. This contrasts with no stimulation in lepromatous cases. The above results do not seem to support the concept proposed by Merklen that lymphocytes from lepromatous leprosy transformed more freely than those of the tuberculoid leprosy with added murine leproma or PHA.

SUMMARY

Measurement of thymidine and uridine incorporation by lymphocytes derived from patients with the two polar types of leprosy (lepromatous and tuberculoid) and of lymphocytes from normal Chinese individuals suggests a depression in immunologic response in untreated lepromatous cases of leprosy and also in cases of tuberculoid leprosy, but to a lesser degree.

RESUMEN

La medida de incorporación de timidina y uridina por linfositos derivados de enfermos con los dos tipos de lepra (lepromatosa y tuberculoidea) y de linfositos de personas chinas normales sugiere una depresión en la respiesta immunológica en casos de lepra lepromatosa non tratados y tambien en casos de lepra tuberculoidea pero con un grado menor.

RÉSUMÉ

La mesure d'incorporation de la thymidine et de l'uridine par des lymphocytes dérivés de malades avec deux types de lèpre (lépromateuse et tuberculoïde) et de lymphocytes d'individues chinois normaux suggère un abaissement dans la réponse immunologique des cas de lèpre lépromateuse non-traités et aussi dans les cas de lèpre tuberculoïde mais à un degré inférieur.

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