Canad. Med. Ass. J.
Sept. 30, 1967, vol. 97

Lymphocyte Transformation by Phytohemagglutinin: I. In Hodgkin's Disease

J. WALLACE THOMAS, M.D., WERNER BOLDT, M.D.,
GAIL HORROCKS, R.T. and BESSIE LOW, A.R.T.,
Vancouver, B.C.

IN recent years evidence has been produced to show that the small lymphocyte plays a major role in "cell-fixed" antibody reactions, such as homograft rejection[1] and delayed hypersensitivity.[2, 3] These reactions have been investigated in patients with Hodgkin's disease and it has been demonstrated that about 50% of such patients will retain homografts and even heterografts of skin for longer than normal intervals. This abnormal rejection phenomenon is only partly related to the stage of the disease,[4, 5] although a higher percentage of grafts will take late in the clinical course. The incidence of anergy is high in Hodgkin's disease[6-8] and is most apparent by the low rate of tuberculin reactions. While evidence of impaired immune response has been accumulating, *in vitro* culture techniques have been revealing new avenues of investigation.

In 1955 Rigas and Osgood[9] showed that an extract of a red bean called *Phaseolus vulgaris* would agglutinate red cells rapidly, allowing the white cells to be readily obtained for culture. The bean extract has been named phytohemagglutinin. In 1960 Nowell[10] demonstrated that cultures of peripheral blood cells obtained after phytohemagglutinin agglutination showed mitotic proliferation which he was able to relate to the activity of phytohemagglutinin. It has since been established that it is the small lymphocyte of the peripheral blood that undergoes mitosis.[11] The sequence of events is that these cells agglutinate and then enlarge to resemble blast cells or Türk cells, a process called either "blastogenesis" or "transformation", and finally undergo mitosis. The red cell agglutinating effect and the mitogenic effect of phytohemagglutinin are probably due to separate substances.[12]

In addition to phytohemagglutinin, a variety of antigens are capable of inducing transformation in the lymphocyte. The extact mode of action of phytohemagglutinin remains uncertain, but differs from that of most antigens in both being more rapid and producing a larger number of transformed cells.

The agents that will cause transformation or blastogenesis may be divided into four groups, as reported by Robbins:[13]

1. General mitogenic agents, such as phytohemagglutinin, staphylococcal filtrate and pokeweed extract.[14]

2. Specific antigens—to which the subject has been previously sensitized, such as purified protein derivative tuberculin, vaccinia vaccine and tetanus toxoid, where the lymphocyte response is dependent upon the previous sensitization.

3. Mixed lymphocyte cultures, wherein the mixing of peripheral blood white cells from individuals, other than identical twins, will show a transformation of the lymphocytes, apparently in response to the foreign white cell antigens.

4. Blastogenesis associated with antiserum, wherein the change is the result of culturing white cells with antiserum directed against white cells.

There are three main ways of judging the lymphocytic response to stimulation:

1. Incorporation of ^{3}H thymidine. This is done most accurately by determining the uptake of ^{3}H thymidine by the whole culture, using liquid scintillation counting. It may also be done more laboriously and less accurately by using autoradiography.

2. Transformation of the cell as judged by morphological standards. These results depend to a great extent upon the subjective assessment by the examiner of morphological change in the small lymphocyte.

3. The mitotic index. After a predetermined interval of culture, a stathmokinetic agent (an agent that blocks cell mitosis) such as colchicine is added and the number of mitotic figures accumulated after a certain time is counted and expressed per thousand cells.

During blastogenesis globulins are produced within the lymphocyte which are presumed to represent antibody.[15] It may be that failure of lymphocytes to transform with phytohemagglutinin indicates immunological incompetence. Normal transformation, however, may not mean that a cell is immunologically competent.

From the Department of Medicine, University of British Columbia, and the British Columbia Cancer Institute, Vancouver.

Supported by a grant from the National Cancer Institute of Canada.

Reprint requests to: Dr. J. W. Thomas, Vancouver General Hospital, Vancouver 9, B.C.

The altered immune state in Hodgkin's disease occurs at all stages of the disease and not infrequently is a contributory factor in the death of the patient.

It has also been established that phytohemagglutinin transformation is impaired in lymphocytes from chronic lymphocytic leukemia[16-18] and because of the similarity in the immunological defect between chronic lymphocytic leukemia and Hodgkin's disease, we have studied the phytohemagglutinin-induced transformation and mitotic index of lymphocytes from patients with Hodgkin's disease and have compared our results with other similar studies.[19-21]

METHODS

Lymphocyte Culture Technique

Thirty millilitres of blood obtained by venipuncture was divided equally between two tubes each containing 400 units of phenol-free heparin and allowed to settle at 37° C. at an angle of 60° until the majority of the red cells had sedimented out. This was usually accomplished in about 45 minutes to one hour. The leukocyte-rich plasma was then removed, measured and pipetted into a sterile glass column packed with absorbent cotton which had been previously washed three times in glass-distilled water and dried at 37° C.[22] The remainder of the blood was then spun at high speed and the cell-free plasma removed and incubated for later use in setting up the cell culture.

The column of white-cell-rich plasma was incubated for 30 minutes at 37° C. and then eluted into sterile centrifuge tubes using twice the volume of minimal Eagle's medium (MEM), containing 100 μg. each of penicillin and streptomycin per millilitre. The eluate was centrifuged at 200 g. for 10 minutes and the cells washed in 10 ml. of Eagle's MEM, then re-centrifuged and re-suspended in 4 ml. of medium. In patients with Hodgkin's disease all but two cultures contained over 93% lymphocytes, the exceptions being 69% and 90%. In all other individuals the white cell cultures contained 93-100% lymphocytes.

The white cells were counted and 4-ml. cultures each containing three million lymphocytes were set up in Eagle's complete medium with 20% fetal calf serum. In transformation studies, Difco phytohemagglutinin P 0.02 ml. was added to each tube, while 0.01 ml. of Burroughs Wellcome phytohemagglutinin, which had been reconstituted in 5 ml. of distilled water, was used for the mitotic index studies. All cultures were then incubated for 72 hours at 37° C. The tubes were then spun at 200 g. for 10 minutes, and transformation in the button of cells was studied on cover-slip preparations stained by Jenner-Giemsa. One thousand cells were counted from each culture on two coverslips.

The mitotic indices were determined after cell mitosis was blocked by 0.1 ml. of a 0.02% colchicine solution. The cells were harvested precisely four hours after the colchicine was added. Colchicine solutions were stored at 4° C. and discarded after three weeks. After the four hours of colchicine treatment, the cultures were pipetted into centrifuge tubes and the cells were re-suspended by gentle manipulation. Following centrifugation at 200 g. for 10 minutes, the supernatant was removed except for about 0.5 ml. in which the cells were re-suspended. Two millilitres of a 0.88% saline solution was then added to the suspension and spun at 160 g. for 10 minutes, after which the supernatant was removed. Two millitres of a 0.22% saline solution was then slowly added down the side of the tube and after four minutes at 37° C. the suspension was spun again at 120 g for 10 minutes. All the saline was removed and Carnoy's fixative was added drop by drop to a volume of 12 ml. Cultures were then left at room temperature for 30 minutes, followed by 10 minutes' centrifugation at 160 g. Enough fixative was removed to leave a fine granular suspension. Slides were prepared, stained with Jenner-Giemsa stain and counted. The number of mitotic figures was reported as an index per 1000 cells.

PATIENTS STUDIED

All patients were attending the British Columbia Cancer Institute and the diagnosis of Hodgkin's disease had been confirmed by lymph-node biopsy. Patients either were untreated or had received no treatment for at least one month. The staging of the disease followed the classification described by Gibson[23] and shown in Table I.

TABLE I.—CLASSIFICATION OF HODGKIN'S DISEASE

I	Involvement of a single site or lymphatic region
II	Involvement of two or more adjacent lymphatic regions
III	Involvement of two or more distant lymphatic regions
	Each stage to be separated into two subdivisions:
	(a) with no symptoms of systemic disease
	(b) with symptoms of systemic disease.

In initial studies, the patients were divided into symptomatic and asymptomatic groups. The symptomatic group was in turn divided into those with and those without systemic disease. The following were taken as representing

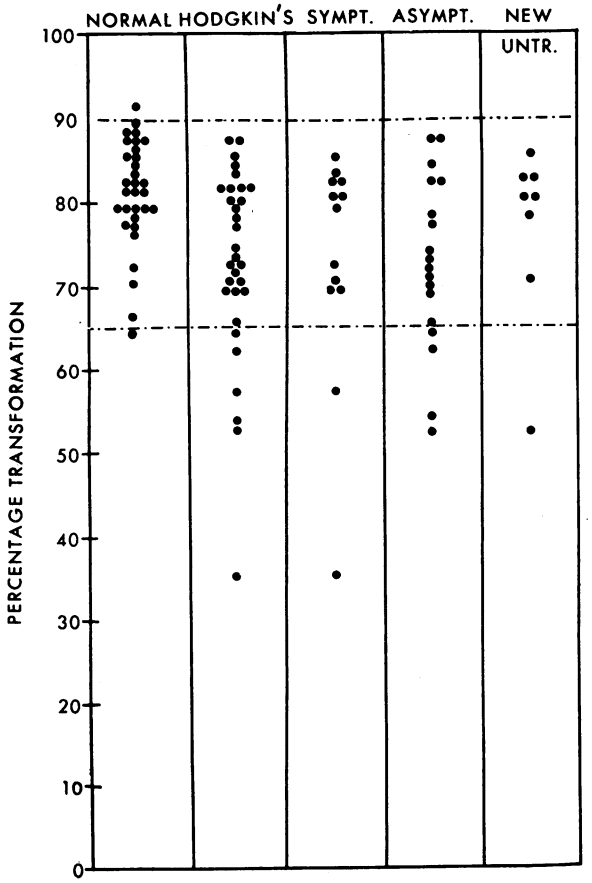

Fig. 1.—Transformation response to phytohemagglutinin of normal and Hodgkin's lymphocytes grown in Eagle's media containing 20% autologous plasma.

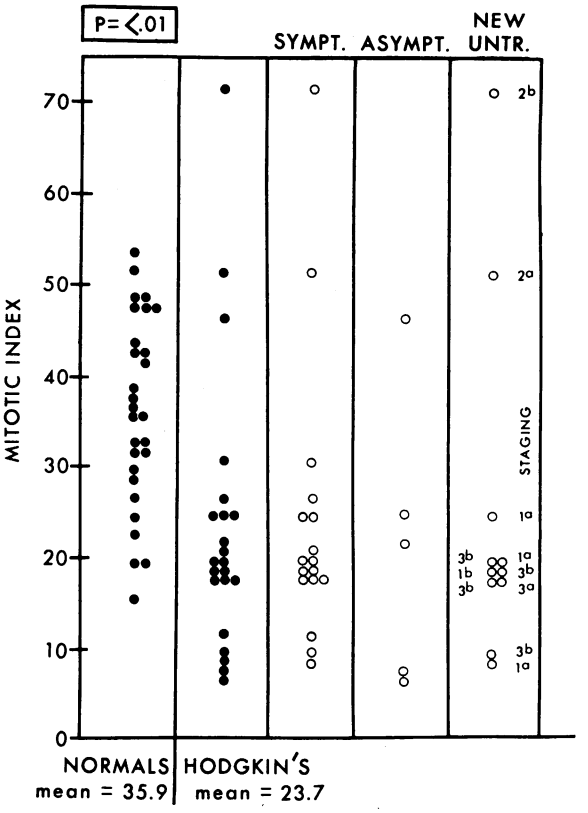

Fig. 2.—Mitotic index of normal and Hodgkin's lymphocytes grown in Eagle's media containing 20% autologous plasma.

systemic involvement where such could be attributed confidently to Hodgkin's disease: anemia, weakness, weight loss, fever, sweating, pruritus, dyspnea and pain.

Normal controls were provided by hospital staff and medical students.

All individuals who had received acetylsalicylic acid or sodium salicylate in the preceding 48 hours were excluded from the study.[24]

RESULTS

The per cent transformation of lymphocytes in normal persons and in patients with Hodgkin's disease is shown in Fig. 1. In this group the cells were grown in Eagle's media containing 20% fetal calf serum. The mean of the normals is 80.7% and that of all patients with Hodgkin's disease 72.8%. The difference, while small, is significant ($p = 0.01$).[25, 26] Further analysis showed the untreated patients to have a mean of 76.2%. Both the symptomatic and asymptomatic had a mean of 72.8%. Seven patients with symptoms for one to six months had a mean value of 78.8% while six patients with symptoms for 6 to 12 months had a mean value of 65.1% transformation. Seven of the symptomatic group had systemic involvement and a mean of 70.1%; the remaining six without systemic involvement showed a mean of 75.5% transformation. Eleven patients treated during the previous 12 months had a mean of 70.0%, while 19 who had no treatment for more than 12 months had a mean of 69.5%. Of the 11 new untreated patients the only one with a result outside the normal range belonged in Stage 1a.

A further group of nine normal persons and 12 patients with Hodgkin's disease was studied. In these the lymphocytes were cultured in autologous plasma. The normal individuals showed a mean transformation rate of 84.4% while the patients with Hodgkin's disease showed a mean rate of 76.4%. This difference is significant at the 0.025 level.

Because judging the lymphocyte response by transformation is so dependent upon subjective interpretation, we next studied the mitotic index of 28 normal persons and 22 patients with Hodgkin's disease. The results, normal 35.9, Hodgkin's 23.7 (Fig. 2), again reveal a difference which is significant ($p = 0.01$). There was no significant difference of the indices between the

new patients and those previously treated, the symptomatic and asymptomatic group, or those with symptoms for less than six months compared to those with symptoms for more than six months. Nor was there any correlation between mitotic index and staging or response to therapy.

The mitotic index of two normal individuals dropped significantly during upper respiratory infections and did not return to normal for six and seven weeks respectively, their normal mitotic indices being 38 and 44 before infection and dropping to 17 and 7 respectively during the infection.

DISCUSSION

The results may be compared with those of others. Aisenberg[19] reported normal transformation in four of 10 patients with Hodgkin's disease with anergy to dinitrochlorobenzene. Hersh and Oppenheim[20] reported markedly reduced lymphocyte response not only to phytohemagglutinin but also to vaccinia, and Schrek[21] reported nearly normal transformation in patients with Hodgkin's disease after phytohemagglutinin.

The present findings are in close agreement with those of Schrek, in so far as transformation is concerned. Transformation is a subjective appraisal to some extent, and the mitotic index seems a more certain method of comparing results. Hersh reports a median mitotic index of 0% for Hodgkin's patients with a normal of 1.5%, while our results show a median mitotic index of 23 (2.3%) for patients with Hodgkin's disease and a median mitotic index of 36 (3.6%) in normal persons.

The results with both fetal serum supplement and autologous plasma are very similar and suggest that the difference between normal persons and patients with Hodgkin's disease are due to a cell abnormality and not a factor in the patient's plasma.

Why the lymphocyte is abnormal in patients with Hodgkin's disease is unknown. Some of the possibilities include general debility, lack of thymic hormone, and viral or other infections.

CONCLUSION

The lymphocytes from some patients with Hodgkin's disease have an impaired response to phytohemagglutinin stimulation which seems unrelated to the clinical staging of the disease in new patients. The response also seems unrelated to previous treatment, the presence of symptoms, the duration of symptoms and the response to therapy. The relationship between this impaired response and the immunological capacity of the lymphocyte remains to be established.

Summary The peripheral blood lymphocytes from patients with Hodgkin's disease were cultured with phytohemagglutinin for three days in Eagle's medium with autologous plasma or fetal calf serum as supplement. The response was compared to that of normal controls by determining the percentage transformation and also by mitotic indices. The mean response by both methods was significantly impaired in patients with Hodgkin's disease. The impairment was due to an abnormality in the lymphocyte. There was no correlation to the clinical staging of the disease in new patients. The response was also unrelated to previous treatment, the presence of symptoms, the duration of symptoms or the response to therapy.

The mitotic index of two normal controls dropped markedly in association with upper respiratory infections believed to be of viral origin.

The relationship between a low mitotic response to phytohemagglutinin and immunological capability remains to be established.

Résumé On a cultivé, avec la phytohémagglutinine, les lymphocytes de sang périphérique prélevé sur des malades atteints de maladie de Hodgkin, pendant trois jours, sur le milieu de Eagle supplémenté par du plasma autologue ou du sérum de fétus de veau.

La réaction lymphocytaire a été comparée à celle de témoins normaux par la détermination du pourcentage de transformation et, également, par les index mitotiques. La réaction moyenne, par les deux méthodes, était notablement altérée chez les malades souffrant de la maladie de Hodgkin. Cette altération était causée par une anomalie dans le lymphocyte. On n'a trouvé aucune relation avec la phase clinique de la maladie chez de nouveaux malades. La réaction n'avait, non plus, aucun lien avec les traitements antérieurs, la présence de symptômes, leur durée ou la réaction du malade au traitement.

L'index mitotique de deux témoins normaux est tombé de façon considérable par suite d'une infection des voies respiratoires supérieures qu'on croit être d'origine virale.

La relation entre une faible réaction mitotique à la phytohémagglutinine et le potentiel immunologique reste encore à établir.

The authors would like to thank Dr. D. M. Whitelaw for his helpful criticism and Drs. R. E. Beck and J. M. W. Gibson for their continuing help. We would also like to acknowledge the International Order of Job's Daughters of British Columbia for their financial assistance in regard to major equipment.

REFERENCES

1. GOWANS, J. L. *et al.*: *Nature (London)*, **196**: 651, 1962.
2. STAVITSKY, A. B.: *Proc. Soc. Exp. Biol. Med.*, **67**: 225, 1948.
3. WESSLEN, T.: *Acta Tuberc. Scand.*, **26**: 175, 1952.
4. MILLER, D. G., LIZARDO, J. G. AND SNYDERMAN, R. E.: *J. Nat. Cancer Inst.*, **26**: 569, 1961.
5. KELLY, W. D. *et al.*: *Ann. N.Y. Acad. Sci.*, **87**: 187, 1960.
6. SOKAL, J. E. AND PRIMIKIRIOS, N.: *Cancer*, **14**: 597, 1961.
7. MILLER, D. G.: *Ann. Intern. Med.*, **57**: 703, 1962.
8. LAMB, D. *et al.*: *J. Immun.*, **89**: 555, 1962.
9. RIGAS, D. A. AND OSGOOD, E. E.: *J. Biol. Chem.*, **212**: 607, 1955

10. NOWELL, P. C.: *Cancer Res.*, **20**: 462, 1960.
11. MACKINNEY, A. A., JR., STOHLMAN, F., JR. AND BRECHER, G.: *Blood*, **19**: 349, 1962.
12. BARKHAN, P. AND BALLAS, A.: *Nature (London)*, **200**: 141, 1963.
13. ROBBINS, J. H.: *Science*, **146**: 1648, 1964.
14. FARNES, P. *et al.*: *Lancet*, **2**: 1100, 1964.
15. ELVES, M. W. *et al.*: *Ibid.*, **1**: 1292, 1963.
16. ELVES, M. W. AND WILKINSON, J. F.: *Exp. Cell Res.*, **30**:
17. SCHREK, R. AND RABINOWITZ, Y.: *Proc. Soc. Exp. Biol. Med.*, **113**: 191, 1963.
18. OPPENHEIM, J. J., WHANG, J. AND FREI, E., III: *Blood*, **26**: 121, 1965.
19. AISENBERG, A. C.: *Nature (London)*, **205**: 1233, 1965.
20. HERSH, E. M. AND OPPENHEIM, J. J.: *New Eng. J. Med.*, **273**: 1006, 1695.
21. SCHREK, R.: *Ibid.*, **275**: 394, 1966.
22. WALKER, R. J. AND FOWLER, I.: *Exp. Cell. Res.*, **38**: 379, 1965.
23. GIBSON, J. M. W.: The treatment of Hodgkin's disease by radiation and chemotherapy. Paper presented at the 10th International Congress of Radiology, Montreal. August 26-September 1, 1962.
24. GANTNER, G. E., JR. AND ZUCKNER, J.: *Arthritis Rheum.*, **8**: 443, 1965 (abstract).
25. CROXTON, F. E.: Elementary statistics, Dover Publications Inc., New York. 1959, p. 87.
26. STEEL, R. G. D. AND TORRIE, J. H.: Principles and procedures of statistics, McGraw-Hill Book Company, New York, 1960, pp. 73, 74 and 433.

Lymphocyte Transformation by Phytohemagglutinin: II. In the Tuberculous Patient

J. WALLACE THOMAS, M.D., SHELDON C. NAIMAN, M.D. and DONNA CLEMENTS, B.Sc., *Vancouver, B.C.*

THE immune response of the body to the tubercle bacillus is apparently accomplished by both circulating "humoral" antibody[1] and by cell-mediated immune reaction.[2] The lymphocyte is responsible for the reaction of delayed hypersensitivity[3, 4] as manifested by the tuberculin skin test and may also be related to the host resistance. Active tuberculosis is frequently found in patients with Hodgkin's disease and there is often reactivation of tuberculosis following steroid therapy. In the first instance there is depression of lymphocyte response to phytohemagglutinin[5, 6] and steroids have been shown to be toxic to lymphocytes.[7] This evidence and the accidental finding of a low mitotic index in a tuberculous patient led us to investigate the response to phytohemagglutinin of the lymphocytes in such patients.

LYMPHOCYTE CULTURE

Mitotic indices were performed as previously described[8] using autologous plasma as supplement.

PATIENTS STUDIED

We confirmed the observation by Gantner and Zuckner[9] that acetylsalicylic acid would depress lymphocyte activity. Normal individuals were given from 2.4 g. to 7.2 g. of acetylsalicylic acid a day for four to five days. The mean pre-therapy mitotic index was 36.5 and the mean post-therapy index was 13.0 ($p = 0.01$)[10, 11] as shown in Fig. 1.

Four categories of tuberculous patients were then studied: (a) newly diagnosed and hitherto

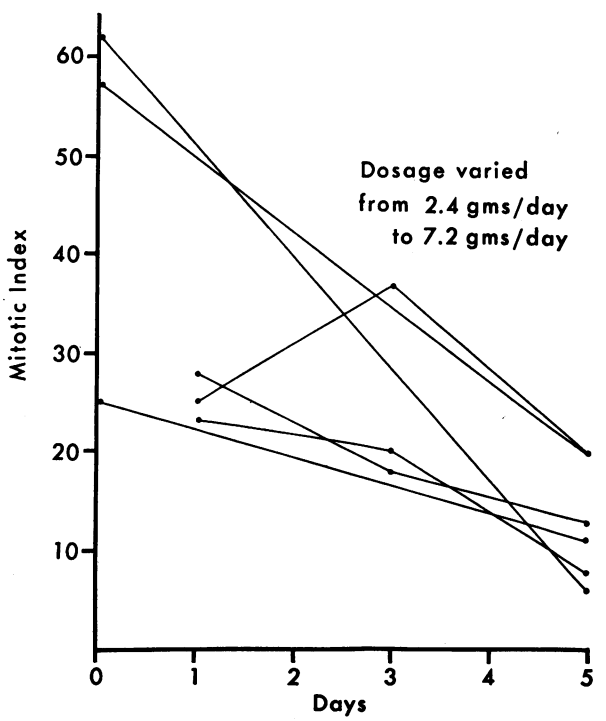

Fig. 1.—The effect of acetylsalicylic acid on the phytohemagglutinin mitotic indices of lymphocytes from normal subjects, grown in 20% autologous plasma supplement. The acetylsalicylic acid was ingested in equally divided dosage over the four- or five-day periods.

From the Department of Medicine, University of British Columbia; the Division of Tuberculosis Control, Provincial Department of Health; and the British Columbia Cancer Institute, Vancouver, B.C.
Supported by a grant from the British Columbia Tuberculosis Society and the Canadian Tuberculosis Association.
Reprint requests to: Dr. J. W. Thomas, Vancouver General Hospital, Vancouver 9, B.C.