4. Baird-Atomic rate meter, model 412, Baird-Atomic, Inc., 33 University Road, Cambridge 38, Mass.
5. Honeywell-Brown Electronik Recorder, Minneapolis-Honeywell Regulator Co., Philadelphia, Pa.
6. Orseal-California Glass Co., 9811 E. Rush St., Del Monte, Calif.
7. Pennsylvania Fluorocarbon Co., Holly St. and Madison Ave., Clifton Heights, Pa.
8. T. W. James, *Ann. N.Y. Acad. Sci.* **90**, No. 2, 550 (1960); G. M. Padilla and T W. James, *Exptl. Cell Res.* **20**, 401 (1960).
9. R. D. Evans, *The Atomic Nucleus* (McGraw-Hill, New York, 1955).

* Special U.S. Public Health Service fellow. Present address: Zoology Department, University of California, Los Angeles 24.

† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

21 August 1963

Immune Response and Mitosis of Human Peripheral Blood Lymphocytes in vitro

Abstract. *Phytohemagglutinin causes cultured lymphocytes to agglutinate, divide, and produce γ-globulin. Most cells are transformed into large lymphocytes, some resembling plasmocytes. Actinomycin D stops γ-globulin production after 2 hours. When specific antigens are added to lymphocytes from sensitized individuals, only some cells undergo morphological transformation, produce γ-globulin, and divide. When cells or cell extracts from an unrelated individual are added to a culture, a similar reaction occurs.*

Phytohemagglutinin, an extract of the kidney bean, *Phaseolus vulgaris,* has been known to be a hemagglutinating agent since 1949 (*1*). In 1959 it was discovered that this extract induces mitosis of human peripheral blood lymphocytes in vitro (*2*). In using a modification of the technique for chromosome study from lymphocytes (*3*), we noticed that mitoses seemed to appear within clumps of lymphocytes. Such clumps did not appear in the absence of the bean extract. The hemagglutinating and mitogenic properties of the extract could be separated, but the latter could not be separated from the ability to agglutinate white cells (*4*). We and others (*5*) observed that during the 72 hours required for mitoses to appear, the vast majority of small lymphocytes were morphologically transformed to large cells, some of which resembled plasma cells. Since, in our method, 80 to 90 percent of the cells at the beginning of culture are small lymphocytes, and since there is no loss of cells during the culture period, it must be these cells which undergo transformation and eventually divide.

The mechanism of action of phytohemagglutinin is not understood. In view of the aforementioned findings, we suspected that some form of immunological reaction occurs in response to the addition of the bean extract to these cells. Pearmain and Lycette, by adding purified protein derivative of tuberculin to the lymphocytes from individuals sensitive to tuberculin, demonstrated that mitoses of peripheral lymphocytes can result from an in vitro immune stimulus (*6*). Bain has reported that DNA is synthesized when a mixture of peripheral blood leukocytes from two unrelated individuals is cultured in a tube (*7*).

We have now studied the possible immune response to phytohemagglutinin and have compared this with the response to specific antigens. All lymphocyte cultures were set up according to the method of Hastings *et al.* (*3*), with the following exceptions: the mitotic arresting agent was vincaleucoblastine (Velban, Eli Lilly) at a final concentration of 7.5×10^{-3} μg/ml (stock, 0.5 μg/ml) for the final 2 hours of culture, and cultures with specific antigens were permitted to grow for 5 days; C^{14}-labeled phenylalanine was used in the incorporation experiments, which were evaluated by standard methods of precipitation with trichloroacetic acid for protein assay and paper electrophoresis.

When phytohemagglutinin was treated by multiple passages through red blood cells until no further hemagglutination occurred, its leukoagglutinating and mitogenic potency was not diminished. This result was independent of the ABO blood type of the donor. When phytohemagglutinin was similarly treated with lymphocytes until no further leukoagglutination occurred, its mitogenic property was lost.

The addition of C^{14}-labeled phenylalanine to the medium at the beginning of the culture period resulted in incorporation of radioactivity into protein found in the supernatant medium when phytohemagglutinin was present. In the absence of phytohemagglutinin only a minimal amount (approximately 20 percent) of such incorporation was found. When actinomycin D(3.2 μg/ml) was added along with the bean extract, the amount of protein produced was the same as that obtained without the addition of extract (see Fig. 1). When the supernatant medium was dialyzed to remove free labeled amino

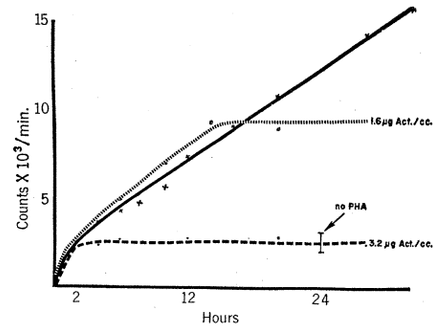

Fig. 1. Incorporation of C^{14}-labeled phenylalanine into protein. (Solid line) Protein production in a culture to which phytohemagglutinin (PHA) was added at time zero. The other two lines represent similar cultures to which phytohemagglutinin and actinomycin D were added at time zero. The vertical bar at 24 hours represents the range of total protein produced by a culture (several experiments) to which no phytohemagglutinin or actinomycin D was added.

acids and the dialysate was subjected to paper electrophoresis, all the radio-activity as read on a chromatogram scanner remained in the γ-globulin region. Since phenylalanine represents approximately 4 percent of human γ-globulin, a calculation based on the radioactivity of the protein obtained by precipitation of the medium with trichloroacetic acid after 24 hours of culture indicates that in the presence of phytohemagglutinin approximately 1 million lymphocytes produced approximately 10 μg of protein as γ-globulin.

Incubation of the cells after the first 24 hours of culture with fluorescein-conjugated rabbit anti-human γ-globulin resulted in specific fluorescence of almost all the lymphocytes in culture. No such fluorescence was observed in the absence of phytohemagglutinin.

In two experiments with lymphocytes from an individual with sex-linked congenital agammaglobulinemia, phytohemagglutinin produced the usual mitotic rate in 72 hours. The usual morphological transformation of cells was observed, but γ-globulin production, as judged by immunofluorescence, could not be detected.

Utilizing lymphocytes from sensitized and nonsensitized individuals, we substituted the following antigens for phytohemagglutinin and examined the cultures for mitotic rate on the 5th day. The antigens and amounts used (per 2×10^6 cells) were purified protein derivative (Parke Davis, 2×10^{-5} to 1.2×10^{-1} mg), diphtheria toxoid (2×10^{-4} mg), pertussis vaccine (Eli Lilly, 5×10^{-3} ml), and penicillin (200

to 20,000 units). In each of these cases several experiments were performed. Lymphocytes from sensitized individuals invariably demonstrated mitosis, while those from nonsensitized individuals did not (Table 1). Two experiments were of particular interest in demonstrating the sensitivity of this method. A 5-month-old child whose lymphocytes did not respond to purified protein derivative was skin tested with first-strength derivative. The skin test was negative, but lymphocytes obtained on the fourth day after the skin test showed a response in vitro (1.2×10^{-3} mg/2×10^{6} cells; mitotic rate, 1.4 percent). Cells from a patient with severe penicillin allergy not only responded to the addition of 50 units of penicillin per 5×10^{5} cells, but in the "control" culture, in which the medium was made up as usual with 10 units of penicillin per milliliter (5×10^{5} cells), a minimal reaction was also observed.

Immunofluorescence studies with cells from sensitized individuals stimulated with purified protein derivative and diphtheria toxoid demonstrated fluorescent staining, but only 5 to 35 percent of the cells fluoresced after 24 hours of culture. Examination of the cells for the morphological transformation as described, revealed that a similar percentage of the cells had undergone this transformation by 5 days.

When lymphocytes from two unrelated individuals were mixed in the same culture tube without phytohemagglutinin, mitoses were observed on the third and fifth days of culture. If one individual's lymphocytes were disrupted by freeze-thawing or hypotonic treatment and then added to another individual's lymphocytes, the same result was obtained. A similar response was observed when skin extract, obtained by freeze-thawing either a biopsy specimen from one individual or the cells from a long-term culture of human skin fibroblasts, was added to the lymphocytes of an unrelated individual.

In a study (*8*) designed to test the hypothesis that there is an autoimmune aspect to infantile eczema, skin extract was prepared from children with this disease and from normal controls. When the extract from an eczematous child was added to his own lymphocytes, a marked mitotic response was observed. The same response was observed when control extract was added to these cells. When the extract from a control was added to his own lymphocytes, no response was seen. Addition of either the extract from the eczema patient or from an unrelated normal person to the cells from a control individual produced a response, but a quantitatively significantly lesser one than that seen in the eczema patient.

Table 1. Results with specific antigens.

Dose per 2×10^{6} cells	Mitotic rate (%)*	Sensitivity of donor
Purified protein derivative of tuberculin		
2×10^{-5} mg	1.2	+
1.2×10^{-3} mg	3.1	+
1.2×10^{-1} mg	2.0	+
1.0×10^{-3} mg	<0.1	−
Diphtheria toxoid		
2.0×10^{-4} mg	1.2	+
2.0×10^{-4} mg	<0.1	−
Pertussis vaccine		
5×10^{-3} ml	1.5	+
5×10^{-3} ml	<0.1	−
Pencillin		
200 units	0.6	+
40 units	0.2	+
200 to 20,000 units	<0.1	−
None	<0.1	

* At least 1000 cells examined.

The utilization of human peripheral blood lymphocytes in the study of the immune response in vitro has many potential applications. These cells are capable of such a response, as is apparent from our findings after the addition of phytohemagglutinin. Our observation that leukoagglutination and mitogenesis are inseparable properties of this substance, but unrelated to hemagglutination, caused us to study the question of whether an immune response was associated with the production of mitoses. The experiments outlined above demonstrate that human lymphocytes are indeed capable of producing γ-globulin in vitro and that almost all the cells are stimulated to such production when phytohemagglutinin is added to the culture medium. There is a concomitant morphological transformation of the vast majority of the cells towards a cell type usually associated with antibody production. The fact that the absence of phytohemagglutinin, as well as the addition of actinomycin D, a substance known to inhibit production of messenger RNA, does not stop γ-globulin production for the first 2 hours of culture would seem to indicate that the lymphocyte contains sufficient messenger RNA in vivo to produce some γ-globulin. Since we obtained identical results after 24 hours if either no phytohemagglutinin or both phytohemagglutinin and actinomycin D were present in the medium, the conclusion may be drawn that phytohemagglutinin stimulates the production of new messenger RNA and new γ-globulin.

The results obtained with specific antigens indicate that an immune response can be obtained from these cells whether the antigen is of the type usually believed to be associated with delayed hypersensitivity or with the production of circulating antibodies. It is of interest that, while phytohemagglutinin causes stimulation of almost all the cells in culture, these specific antigens stimulate only 5 to 35 percent of the lymphocytes. One may presume that only a limited number of lymphocytes in vivo carry the information required to respond to specific antigens. The fact that the great majority of cells respond to phytohemagglutinin requires a different hypothesis for its action than that considering this substance to be a specific antigen. It is possible that phytohemagglutinin may, analogous to an antibody, bind to some substance on the surface of the lymphocyte, thereby causing the cells to clump and nonspecifically stimulating the lymphocytes to produce whatever γ-globulin they may be capable of producing. This same membrane phenomenon may also be responsible for the induction of mitosis. This hypothesis is supported by the findings in the agammaglobulinemics and by some preliminary results in which lymphocytes from individuals producing abnormal γ-globulins such as macroglobulins and myeloma proteins were used. These individuals' cells, when stimulated with phytohemagglutinin, produced the abnormal γ-globulin. Although the same lymphocytes appear to undergo transformation and produce γ-globulin, γ-globulin is not essential for transformation or for mitosis, as demonstrated in the agammaglobulinemic patients. It appears that γ-globulin production is a secondary response occurring, when possible, as a consequence of some process involved in lymphocyte transformation.

The response of lymphocytes to cells or cell extracts from unrelated individuals would seem to indicate that the histocompatibility reaction observed as homograft rejection may be detectable in vitro. Since preliminary studies indicate that the cells from monozygous twins do not react with each other, this method may provide a useful tool for

finding compatible graft donors and for the study of the genetics of histocompatibility in man. Similarly, the findings in the eczema experiments provide hope that this method may be useful in the study of autoimmune disease (*9*).

KURT HIRSCHHORN, FRITZ BACH
ROSELYN L. KOLODNY
I. LESTER FIRSCHEIN, NEMAT HASHEM
Department of Medicine, New York University School of Medicine, New York 16

References and Notes

1. J. G. Li and E. E. Osgood, *Blood* **4**, 670 (1949).
2. D. A. Hungerford, A. J. Donnelly, P. C. Howell, S. Beck, *Am. J. Human Genet.* **11**, 215 (1959).
3. J. Hastings, S. Freedman, O. Rendon, H. L. Cooper, K. Hirschhorn, *Nature* **192**, 1214 (1961).
4. R. L. Kolodny and K. Hirschhorn, in preparation.
5. K. Hirschhorn, R. L. Kolodny, N. Hashem, F. H. Bach, *Lancet* **II**, 305 (1963); W. H. Marshall and K. B. Roberts, *Quart. J. Exptl. Physiol.* **48**, 146 (1963).
6. G. Pearmain, R. R. Lycette, P. H. Fitzgerald, *Lancet* **I**, 637 (1963).
7. B. Bain, M. Vas, L. Lowenstein, *Federation Proc.* **22**, 428 (1963).
8. N. Hashem, S. Snyderman, L. E. Holt, Jr., K. Hirschhorn, *Lancet* **II**, 269 (1963).
9. We thank Susan Verbo for her technical assistance. Work supported by grants from the American Heart Association, the U.S. Public Health Service (GM08488), the New York City Health Research Council (U-1030), and the Association for the Aid of Crippled Children. One of us (K.H.) is an established investigator of the American Heart Association.

19 August 1963

Bipolar Planarians in a Stock Culture

Abstract. *Four long bipolar planarians were discovered in a stock culture of asexual* Dugesia dorotocephala. *Such worms have not been previously reported. Eyespots, auricles, and responses to stimuli were normal in each head-end, and functional pharynges were present. Worms in the stock culture had not been subjected to temperature variations, chemicals, or other experimentation. It is suggested that the axial gradient was effectively flattened by a normal head-inhibiting factor being unable to diffuse to the posterior end.*

Bipolar planarians have been produced from extremely short segments (*1, 2*) and, from somewhat larger segments, by the use of chemicals such as lithium chloride plus thiocyanate, colchicine, and deacetylmethylcolchicine (*3, 4*). No reports have been published of the natural occurrence of bipolar heads in long, mature planarians not subjected to experimental conditions.

In the summer of 1962, three long bipolar planarians were discovered in a stock culture of asexual *Dugesia dorotocephala*. The worms had been kept in a constant temperature laboratory for 3 years, and had not been exposed to chemicals or other form of experimentation. In the spring of 1963, a fourth bipolar planarian was discovered in the same group. This animal measured about 30 mm in length (Fig. 1); one head was slightly larger, but both heads were normal in appearance. A functional pharynx was present in each end.

Approximately 6 weeks after the animal was found, fission occurred. The fission plane was in the usual position behind the pharynx associated with the larger head. The long mid-piece, which remained attached to the smaller head, was held more or less curled over the back of the worm as it glided about. Viewed with a microscope, no injury of any type was apparent on either worm. Within a short time, the piece with the larger head regenerated a tail, and thereafter gave rise to several normal fission products.

Ten days after fission, the longer piece again became bipolar. Within a few days the two heads were the same size, each with well-developed eyespots and auricles. It was no longer possible to distinguish which was the newer regenerate. At all times, the two head-ends reacted as two individuals, whether ingesting food, crawling over each other, or starting off in separate directions. The connecting mid-piece appeared to be under the control of neither head, but apparently offered no obstacle to movement.

According to Child's theory, axial metabolic gradients constitute the basis of polarity, and bipolarity in short segments of planarians develops because little or no gradient has been established (*2*). Flickinger demonstrated, in intact planarians, an axial anterior-posterior gradient of incorporation of $C^{14}O_2$ and C^{14}-labeled glycine into proteins, and showed that both deacetylmethylcolchicine and chloramphenicol, known inhibitors of protein synthesis, could obliterate this gradient, and were effective in producing reversed polarity and bipolar development (*4*). Apparently, for bipolarity to occur, the slope of the gradient must be flattened by some means. Since, in this case, neither shortness of segment, nor chemicals, can be considered, it would appear that either the excessive length or the somewhat inert mid-piece, or both, must have been a major factor in the differentiation of the posterior head.

Fig. 1. Bipolar planarian from a stock culture.

That distance may have some effect is shown by Flickinger's report of a slight increase in the tail region of the axial gradient of incorporation of C^{14}-labeled glycine (*4*). Further evidence is provided by Lender's demonstration that in planarians, the differentiation of another brain can be inhibited either by a brain present in the animal, or by a crude homogenate of heads added to water in which decapitated planarians are cultured. This inhibitory substance is diffusible, and its effect decreases with distance (*5*).

It appears probable that the formation of the second head was due to an effective flattening of the gradient, with resultant escape from anterior dominance, and that the means of accomplishing this was the inability of a diffusible inhibiting substance to traverse the distance required. In the absence of such an inhibitor, a secondary head could form in the usual manner (*6*).

MARIE M. JENKINS
Department of Biology, Madison College, Harrisonburg, Virginia

References and Notes

1. T. H. Morgan, *Biol. Bull.* **3**, 132 (1902).
2. C. M. Child, *Individuality in Organisms* (Univ. of Chicago Press, Chicago, 1915), pp. 99–100.
3. O. Rulon, *Physiol. Zool.* **31**, 231 (1948); M. A. McWhinnie, *Biol. Bull.* **108**, 54 (1955); H. Kanatani, *J. Fac. Sci. Univ. Tokyo, Sect. IV* **8**, 253 (1958).
4. R. A. Flickinger, *Growth* **23**, 251 (1959).
5. Th. Lender, *Bull. Soc. Zool. France* **81**, 192 (1956); *J. Embryol. Exptl. Morphol.* **8**, 291 (1960).
6. I thank R. A. Graves for photographing the planarian.

14 October 1963