Lymphocyte Transformation with Phytomitogens in Leprosy

Marian Ulrich, Brunilda de Salas and Jacinto Convit²

In recent years, several reports have appeared concerning the transformation of lymphcytes from leprosy patients by phytohemagglutinin. With the exception of papers by Sheagren et al (11, 12), these investigations indicate lower transformation than normal in this disease, particularly with lymphocytes from patients with active lepromatous leprosy (3, 5, 8, 10). This impaired response has been interpreted as one of the manifestations consistent with an apparent generalized depression of delayed hypersensitivity in leprosy.

The purpose of the present study has been to investigate the response of lymphocytes from leprosy patients to another widely-used phytomitogen, pokeweed extract. During the course of the work, comparative studies were made of the lymphocytic transformation by pokeweed and by phytohemagglutinin. The evaluation of transformation, which was based on morphologic criteria, was greatly facilitated by staining with acridine orange to differentiate between lymphoblasts or transformed cells and unaltered cells. The results are discussed in terms of their relation to the apparent immunological defect in lepromatous leprosy.

MATERIALS AND METHODS

Phytomitogens. Pokeweed mitogen (PWM) was obtained from Grand Island Biological Co., New York. It was used in the concentration recommended by the supplier; 0.1 ml of a 1:10 dilution of the original volume per milliliter of cell suspension.

Phytohemagglutinin-P (PHA) was obtained from Difco Co., Detroit, Michigan, and was used in a concentration of 20 μg per millimeter of cell suspension.

Lymphocyte cultures. Twenty milliliters of heparinized blood (10 units of preservative-free sodium heparin per milliliter) were allowed to sediment at 37° C for 30 to 60 minutes. When the sedimentation of red blood cells was nearly complete, the leucocyte-rich supernatant fluid was decanted, diluted with an equal volume of McCoy 5-A medium (Grand Island Biological Co.) containing 100 units of penicillin and five units of heparin per milliliter, and incubated in a prescription bottle placed on its flat surface at 37° C for one hour. The semi-purified lymphocytes were counted and diluted to a concentration of 1 x 106 lymphocytes per milliliter with McCoy 5-A medium. These suspensions contained 12% to 20% autologous plasma, depending upon the dilution required. The cell suspensions were distributed in volumes of two milliliters per Leighton tube and incubated at 37° C in an incubator containing a 95% air-5% CO_2 mixture for three to five days. Usually three tubes with PHA, three with PWM, and three controls were prepared from each cell suspension.

Determination of percentage of transformation. On the day of harvest, the cells were separated by centrifugation for ten minutes at 1000 rpm then washed with two milliliters of phosphate-buffered saline, pH 7.2. The supernatant fluid was decanted after centrifugation and the cells were suspended in the small volume of fluid which remained. Slides were prepared by spreading a drop of this suspension uniformly over an area about 1.5 cm in diameter. After gentle warming to hasten drying, the slides were fixed in methanol for ten minutes and stained with acridine orange. The following modification of the method of Von Bertalanffy and Bickis (15) was used: fixed slides were passed rapidly through 80%, 70% and 50% ethanol, distilled water, 1% acetic acid and distilled water. They were stained for three minutes in 0.01% acridine orange buffered to pH 6.0 with M/15 phosphate buffer, rinsed for one minute in the same buffer, and decolorized with 0.1 N CaCl₂ for two minutes. After final rinsing for one minute in phosphate

¹ Received for publication June 28, 1971. ² M. Ulrich, Ph.D., Immunologist; B. de Salas, Research Assistant; and J. Convit, M.D., Director, Laboratrio de Microbiología Experimental, División de Dermatología Sanitaria, Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela.

buffer, a cover slip was mounted with the same buffer and the slides were examined with a fluorescent microscope. Five hundred to 1000 lymphocytes were counted on each slide in order to determine the percentage of transformed cells.

Classification of disease state. The classification of the patients according to the clinical form of disease was determined by several criteria, which included clinical manifestations, bacteriologic examination of smears, histologic examination of biopsies, and cutaneous response at three weeks to the injection of 0.1 ml of standard integral lepromin, prepared according to the method of Mitsuda-Hayashi-Wade and containing 160 x 10⁶ bacilli per milliliter.

RESULTS

Table 1 presents the results obtained when transformation was compared after four days of incubation with PWM in groups of treated ambulatory lepromatous leprosy (LL) patients, untreated (LL) patients and normal controls. No significant difference was noted between the treated LL and normal groups; the percentage of transformation with the untreated LL group was somewhat reduced, significant at the level .02 > P > .01. It should be noted that the total processing time of the lym-

TABLE 1. Lymphocytic transformation with pokeweed mitogen in treated and untreated lepromatous leprosy and controls.

	ous leprosy	Lepromate
Normal controls	Untreated	Treated
36	9	25ª
38	20	29
40	21	30
42	24	30
42	24	35
52	28	36
53	41	43
55	42	48
59	42	56
100 million - 10	59	59
		59
		64
46	31	Av. 43

* Percent transformation at four days.

TA	BLE	2.	L_{1}	ymphocy	tic	transf	ormati	on
with	pok	ewe	ed	mitogen	and	l with	PHA	in
untre	ated	llep	ros	<i>y</i> .				

	Percent trans	ercent transformation		
Group	Pokeweed	PHA		
Borderline	58	59		
leprosy	59	56		
1	60	18		
Tuberculoid	39	64		
leprosy	48	51		
	9	64		
Lepromatous	24	29		
leprosy	24	57		
	41	40		
	42	47		
	59	90		
	38	64		
Control	52	54		
	53	57		
	55	74		

phocyte cultures from the untreated LL group was somewhat longer than for the other two groups, since these patients were all hospitalized at some distance from the laboratory. In this and subsequent aspects of the study, the concentration of autologous serum present was not correlated with the percentage of transformation.

Table 2 presents the comparison of groups of patients with untreated leprosy and controls when both PWM and PHA were used to provoke transformation. Though the average transformation with both phytomitogens in the group of LL patients is somewhat lower than in the control group, this reduction is not highly significant.

In an effort to control some of the variables presented by the transportation of blood samples and to see if a more pronounced depression of transformation might be observed in persons with severe, bacteriologically-active LL of long duration, 24 patients from the Cabo Blanco Leprosarium were brought to our laboratory for blood extraction. Cultures of lymphocytes from each patient were examined



FIG. 1. Responses at 3, 4, and 5 days to pokeweed mitogen by lymphocytes from lepromatous leprosy and normal controls.

after three, four, and five days of incubation with PWM and PHA. These results are presented in Figures 1 and 2. It is apparent that there were no significant differences in the responses to either of these phytomitogens when lymphocytes from these patients were compared with lymphocytes from normal persons.



FIG. 2. Response at 3, 4, and 5 days to PHA by lymphocytes from lepromatous leprosy and normal controls.

As we have briefly reported previously (14), the use of acridine orange enormously facilitates the identification of lymphoblasts. These cells, which are active metabolically, are characterized by their increased size, relatively large cytoplasm to nucleus ratio, and very pronounced reddish-orange staining of the cytoplasm with acridine orange. Preliminary results indicate that 0.1% ribonuclease abolishes this characteristic cytoplasmic staining. Small lymphocytes are characterized by an extremely narrow, slightly reddish band of cytoplasm and the nuclear material is green. Macrophages, which were rarely seen in these preparations, are easily distinguished from lymphoblasts by structural features as well as the very slight extent to which the cytoplasm of the former is stained red in comparison with the lymphoblasts.

The fluorescence is stable for several hours at room temperature if protected from light, and for many months if kept below freezing. The slides are easily decolorized by a brief wash in 50% ethanol and can then be restained by other methods.

DISCUSSION

Several observations have been cited as evidence that there is a generalized depression of cellular hypersensitivity in lepromatous leprosy. The frequency or intensity of positive skin reactions to tuberculin, oidiomycin, and other antigens is reportedly lowered in these persons (1, 2, 7). It has been reported that a smaller percentage than normal can be sensitized to picryl chloride or dinitrochlorobenzene (1, 16). Rejection of skin grafts reportedly is retarded in lepromatous leprosy (5). Finally, several authors have reported a reduced response to phytohmagglutinin (3, 5, 8, 10). Though PHA-induced lymphocyte transformation is clearly an indirect measure of immune capacity, lowered or delayed responses to PHA have been reported in Hodgkin's disease, sarcoidosis, and chronic lymphocytic leukemia, where there is a clear association with generalized impairment of delayed hypersensitivity.

Most evidence suggests that the postulated nonspecific depression of cellular immunity in lepromatous leprosy is a consequence of the infection itself; adequate treatment is accompanied by recovery of skin reactivity. However, the possibility exists that people who are healthy but persistently Mitsuda-negative might be poor responders by several immunologic criteria; thus studies of this group of persons with skin test antigens, phytomitogens, and contact sensitizers would be of interest. With this long-range objective in mind, we began a study of the response of lymphocytes from lepromatous patients to pokeweed mitogen, which has not been previously studied in leprosy, in order to compare its activity with that reported for PHA. This preliminary study revealed a relatively small depression of lymphocyte transformation in untreated lepromatous leprosy, not comparable to the depression reported by most authors in studies with phytohemagglutinin.

There is good evidence that PHA and PWM react with somewhat distinct populations of lymphocytes. The percentage of lymphocytes reacting with PHA is normally higher (⁶), and the chemical nature of the combining sites is different (⁹). The morphology of the transformed cells also differs in some aspects (⁶). Therefore, a number of lymphocyte suspensions were treated with both PHA and PWM, to determine if a markedly reduced response to PHA might occur in spite of a relatively normal response to PWM. This postulated difference was not observed.

The possibility existed that the untreated LL patients we examined were not representative of the types of patients examined by other authors, since in the majority of our initial cases the infection was neither severe nor of long duration. However, the examination of lymphocytes from patients with severe, bacteriologically-active lepromatous leprosy of long duration failed to reveal differences in the response to either PHA or PWM when compared to a normal control group.

We cannot offer an explanation for the differences observed between our results and those of other authors. The method described here is not basically different from that used by others, and the morphological criteria were those used in most of the studies reported in leprosy. It should be noted that Sheagren *et al* also reported normal transformation in one of the earliest studies reported $(^{11})$.

We wish to emphasize that investigations by Convit et al (4) present data in which generalized nonspecific defect in a delayed hypersensitivity could not be demonstrated in lepromatous leprosy. These data, together with the observations presented in this paper, suggest that the basic immunologic defect in lepromatous leprosy, whether it be pre-existing or a result of some process such as tolerance or immune deviation, may be quite highly specific for Mycobacterium leprae. Nonspecific depression of cellular hypersensitivity would not seem to be a constant observation, but rather a variable condition conceivably modified by genetic and environmental factors. Of considerable interest, and currently under study by one of the authors of this paper (Convit), is the examination of lymph nodes from persons with various clinical manifestations of leprosy. Though by clinical criteria there seems to be no doubt about our classification of lepromatous leprosy, perhaps the profound changes reported in lymph node structure by Turk and Waters (13) are not characteristic of the cases we have studied.

Acridine orange staining for morphologic evaluation of lymphocyte transformation apparently has not been previously reported. The procedure is rapid and identification of transformed cells is simple even for persons with very little training in cell identification. We would recommend this procedure as a useful clinical and investigative method.

SUMMARY

Lymphocyte transformation was studied using the phytomitogens, phytohemagglutinin and pokeweed extract. In a small group of untreated patients with lepromatous leprosy, some reduction in the percentage of transformation was noted. In a larger group of persons with severe, bacteriologically-active lepromatous leprosy of long duration, cultures at three, four and five days showed no significant differences

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with either phytomitogen when compared to control cultures from normal individuals.

Morphological evaluation of transformation was determined by staining with acridine orange, which offers a number of advantages for this purpose.

RESUMEN

Se estudió la transformación de linfocitos utilizando los fitomitógenos fitohemaglutinina y extracto de fitolaca. En un pequeño grupo de pacientes con lepra lepromatosa no tratados se observó una ligera reducción del porcentaje de transformación. En un grupo mayor de personas con lepra lepromatosa severa, bacteriológicamente activa, de larga duración, los cultivos a los tres, cuatro y cinco días no nostraron diferencias significativas con ninguno de los dos fitomitógenos, cuando se compararon con cultivos controles obtenidos de individuos normales.

La evaluación morfológica de la transformación se determinó por medio de tinción con naranja de acridina, la cual ofrece una serie de ventajas para este propósito.

RÉSUMÉ

On a eu recours aux phytomitogènes, à la phytohémagglutinine et à des extraits de graines (pokeweed) pour étudier la transformation lymphocytaire. Dans un petit groupe de malades non traités et atteints de lèpre lépromateuse, on a observé une certaine réduction dans le pourcentage de transformations. Dans un groupe, plus considérable, d'individus souffrant d'une lèpre lépromateuse grave, active du point de vue bactériologique et de longue durée, les cultures au troisième au quatrième et au cinquième jours n'ont pas révélé de différences à la suite de l'addition de l'un ou l'autre de ces agents phytomitogènes, lorsqu'on les comparait aux cultures témoins provenant d'individus normaux.

L'évaluation morphologique de la transformation a été déterminée au moyen d'une coloration par l'acridine orange. Cette technique présente un certain nombre d'avantages pour ce genre d'études.

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REFERENCES

- BULLOCK, W. E., JR. Depression of the delayed-type allergic response in leprosy. Clin. Res. 14 (1966) 337.
- BULLOCK, W. E., JR. Studies of immune mechanisms in leprosy. I. Depression of delayed allergic response to skin test antigens. New Eng. J. Med. 278 (1968) 298-304.
- 3. BULLOCK, W. E., JR. and FASAL, P. Impairment of phytohemagglutinin (PHA) and antigen-induced DNA synthesis in leucocytes cultured from patients with leprosy. Internat. J. Leprosy 36 (1968) 608.
- 4. CONVIT, J., PINARDI, M. E. and ARIAS ROJAS, F. Some considerations regarding the immunology of leprosy. In press, Internat. J. Leprosy.
- DIERKS, R. E. and SHEPARD, C. C. Effect of phytohemagglutinin and various mycobacterial antigens on lymphocyte cultures from leprosy patients. Proc. Soc. Expt. Biol. Med. 127 (1968) 391-395.
- DOUGLAS, S. D., HOFFMAN, P. F., BORJE-SON, J. and CHESSIN, L. N. Studies on human peripheral blood lymphocytes in vitro. III. Fine structural features of lymphocyte transformation by pokeweed mitogen. J. Immunol. 98 (1967) 17-30.
- LEIKER, D. L. Studies on the lepromin test. IV. Influence of leprosy on the reactions to lepromin, tuberculin and the "875 bacillus" suspension. Internat. J. Leprosy 29 (1961) 496-501.
- PARADISI, E. R., BONAPARTE, Y. P. DE, and MORGENFELD, M. C. Blasts in lepromatous leprosy. Lancet 1 (1968) 308-309.
- RIEKE, W. O., ed. Proceedings of the Third Annual Leucocyte Culture Conference. (1969) Appleton-Century-Crofts, New York, N.Y.
- RODRIQUEZ, E., BONAPARTE, Y. P. DE, and MORGENFELD, M. C. Cultivo de linfocitos en enfermos con lepra lepromatosa (Comunicasión previa). Leprología 12 (1967) 61-63.
- SHEAGREN, J. N., BLOCK, J. B., TRAUT-MAN, J. R. and WOLFF, S. M. Immunologic reactivity in leprosy. Clin. Res. 15 (1967) 300.
- SHEAGREN, J. N., BLOCK, J. B., TRAUT-MAN, J. R. and WOLFF, S. M. Immunologic reactivity in patients with leprosy. Ann. Intern. Med. 70 (1969) 295-302.
- TURK, J. L. and WATERS, M. F. R. Immunological significance of changes in lymph nodes across the leprosy spectrum. Clin. Expt. Immunol. 8 (1971) 363-376.

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- ULRICH, M., SALAS, B. DE, and CONVIT, J. Transformación linfocitaria con fitolaca en la lepra. Acta Científica Veneaolana 21 (1970) 58.
- (1970) 58.
 15. Von BERTALANFFY, L. and BICKIS, I. Identification of cytoplasmic basophilia (ribonucleic acid) by fluorescence micros-

copy. J. Histochem. Cytochem. 4 (1956) 481-493.

 WALDORF, D. S., SHEACREN, J. N., TRAUT-MAN, J. R. and BLOCK, J. B. Impaired delayed hypersensitivity in patients with lepromatous leprosy. Lancet 2 (1966) 773-775.