

PM-AAA-536

YD-AAA-536-A

AGENCY FOR INTERNATIONAL DEVELOPMENT

WASHINGTON DC 20523

DATE: _____

8/4/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5. 141
PR # 2
PR 7/1/88 - 12/30/88

Attachment

PN-ABA-536

57391

5.141

PROGRESS REPORT NO 2

DEVELOPMENT OF AN IMMUNOPEROXIDASE TEST FOR EARLY DIAGNOSIS
OF ACUTE REACTIONAL STATES IN LEPROSY

USAID/PSTC GRANT NO: 936-5542-G-00-5044-00

SUBMITTED BY

Dr. Choti Theetrantont, M.D.,
Chairman,
Department of Pathology

and

Dr. Kanthorn Thanprasert, M.D.
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Chiang Mai 50002

and

Dr. D.M. Scollard, M.D., Ph.D.
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University of Hawaii
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Honolulu, Hawaii

Rec'd in SCI. AUG 20 1986

PROJECT PROFILE

Country: Thailand

Grant No: 936-5542-G-00-5044-00

Program: Program on Science and Technology Cooperation

Project Title: Development of an Immunoperoxidase Test for
Early Diagnosis of Acute Reactional States
in Leprosy.

Project Leader: Choti Theetranont, M.D.

Organization: Department of Pathology,
Faculty of Medicine,
Chiang Mai University

Co-Investigators: See Below

Project Consultants: Trevor Smith, M.D., Barbara Mills, M.D.
McKean Rehabilitation Institute for
Leprosy Patients.

Authorized Officer: Choti Theetranont, M.D.

Total Project Budget: US\$ 107,400

Project Duration: 1 July 1985 to 30 June 1987

Report Period: Second Reporting Period 1 January to 30 June 1986

Budget Allocation for
This Period: Baht 564,975.00

2

PROGRESS REPORT

USAID/SCI Office of Science Advisor Number 5.141

Title: "Development of an Immunoperoxidase test for Early Diagnosis of Acute Reactional States in Leprosy"

Project Period: Jan 1, 1986 - June 30, 1986. Second reporting period.

Investigators:

David M. Scollard, M.D., Ph.D., Department of Pathology, University of Hawaii School of Medicine;

Choti Theetanont, M.D., Department of Pathology, Chiang Mai University, Chiang Mai, Thailand;

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Sanki Mahonkornkeyoon, Ph.D., Department of Clinical Immunology, Chiang Mai University, Chiang Mai, Thailand;

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Limkorn Chakrasert, M.D., Department of Pathology, Chiang Mai University, Chiang Mai, Thailand.

Publications resulting from this work:

Scollard, D.M., Rangdaeng, S., and Kenney, R.T. (1985) "Evolution of cutaneous immune responses in tuberculosis and leprosy" Lab. Invest. 2: 60.

Kenney, R.T., Rangdaeng, S., and Scollard, D.M. "Cellular Kinetics of the Human Immune Response to PPD Using Suction-Induced Skin Blisters: An Immunocytochemical Study." (Submitted to J. Immunol. Methods)

Scollard, D.M., Rangdaeng, S., and Kenney, R.T. 1985 "Kinetic patterns of cellular response to repeated tuberculin testing in healthy responders and in subclinical tuberculosis; Correlates to the 'Booster effect'" (Submitted to Clin. Immunol. Immunopath.)

Tung, K.S.K., Nelson, K., Rubin, L., Wagner, D., Umland, E., Schauf, V., Scollard, D., Vithayasai, P., Vithayasai, V., Worobec, S., Smith, T., and Suriyanond, V. 1985 "Serum soluble interleukin-2 receptor levels in leprosy patients" Presented at the US-Japan Leprosy Panel, Aug, 1985, Bethesda, Md. (Inf. Immun., in press)

Manuscripts in preparation:

Rangdaeng, S., Scollard, D.M., Suriyanond, V., Makonkawkeyoon, S., and Theetranont, C. "Mononuclear cell subsets from uncomplicated cutaneous lesions of leprosy in man: An immunocytochemical study using suction-induced skin blisters".

Scollard, D.M., Tung, K.S.K., Rubin, L., Wagner, D., et al "Soluble interleukin-2 receptor levels in fluid from suction-induced blisters of cutaneous leprosy lesions"

Suriyanond, V., Scollard, D.M., Rangdaeng, S., et al "The Clinical Course of Simple and Recurrent Erythema Nodosum Leprosum: A Clinical Study"

Scollard, D.M., Rangdaeng, S., Suriyanond, V., Theetranont, C., Makonkawkeyoon, S., Arnold, R., and Smith, T. "Increased Numbers of IL-2 Receptor-Bearing Cells in the Epidermi Characterize Leprosy Reversal Reactions"

Project Objectives:

Unchanged;

1. To determine, in skin lesions of leprosy patients, changes in the distribution and/or ratio of lymphocyte and monocyte subpopulations which accompany acute reactions in leprosy, using monoclonal antibodies.
2. To develop a non-invasive "skin window" method of monitoring lymphocyte and monocyte changes that will enable early identification of patients with a high risk of impending reaction.

Progress of Project Activities

1. Both clinical and laboratory work have proceeded smoothly. The volume of patients to be studied has grown progressively, since several patients are now being followed and studied sequentially, in addition to the accumulation of new patients. To enable us to handle the increased load, an additional technician has been hired.

Dr. Rangdaeng has taken a well-deserved pathology residency position at Baylor College of Medicine, Houston, Texas, and Dr. Choti has recruited Dr. Kamthorn Thanmaprasert to assume Dr. Rangdaeng's responsibilities in this project.

The Research Institute Institute for Health Sciences (RIHES), Chiang Mai University, has received a grant from the World Health Organization for the establishment of a Tropical Disease Research Unit within the Institute, one facet of which is leprosy research. Our project staff are very much involved in initiating this effort, and will work closely with RIHES staff to integrate our work with the new project in areas of natural overlap. This will also provide one means for the continuation of the present work after the grant from USAID has expired. In addition, as a result of findings discussed below, we are planning to submit another application to the AID/SCI program in order to pursue some new possibilities of more simplified clinical diagnostic techniques.

2. At the time of this writing 91 patients have been studied, many of them on multiple occasions (one patient having been studied six times, before, during, and after multiple reactions). This represents an increase of 40 patients in the six months elapsed since the last report; the majority of these have been new patients without reactions, who are now being followed. Fifteen inactive patients have been studied as controls for the results seen in active patients.

Our technical methods are now quite well standardized, and no important modifications are being tested in the field, although some new approaches are being tested in Honolulu for possible introduction in Thailand, if and when they are found to be satisfactory.

The panel of antibodies currently being used in Chiang Mai is:

Leu-4	all T cells
Leu-3	T-helper cells
OKT-8	T-suppressor cells
IL-2R	Activated cells bearing the IL-2 receptor
OKT-6	Langerhans cells

Results

Findings in three areas appear to be the most interesting and important thus far: The numbers and distribution of IL-2-receptor bearing cells, the clinical features of ENL (which may be correlated with the findings on IL-2R), and the measurement of antibody and IL-2R in the blister fluid.

IL-2 Receptors. Lymphocytes which have been stimulated by specific antigen, and which will proliferate as a result, also produce the immunoregulatory substance designated "Interleukin 2" (IL-2). For IL-2 to stimulate other cells, and thus amplify the immune response, other lymphocytes must express increased amounts of IL-2 receptors (IL-2R) in their surface membranes. Identification of such IL-2R+ cells, therefore, is a measure of immunologic stimulation which is most directly related to initiation of cell-mediated immunity.

We have observed that in skin biopsies from patients with reversal reactions, the number of IL-2R+ cells is increased (Table 1 and Fig. 1). This increase was not seen in a patient with a "downgrading reaction", nor in a patient who had been treated recently with corticosteroids. Moreover, the number of IL-2R+ cells in the epidermis declined with treatment in one patient who had been biopsied just at the outset of his reaction, and again three weeks into his treatment.

Of special interest is that the increased number of IL-2R+ cells is observed in the epidermis, rather than in the dermis where the leprosy bacilli and associated inflammatory infiltrate are located. We believe that this may be related to increased functional activity on the part of Langerhans cells, the principal antigen-presenting cells in the skin, which are also located in the epidermis. We are therefore focussing specifically on the identification and enumeration of Langerhans cells in our biopsies and blister fluids.

Patients with uncomplicated leprosy or with ENL had much smaller numbers of IL-2R+ cells in the epidermis (Fig. 1 and Table 1). One individual, however, had numbers of these cells midway between the ENL and Reversal Reaction patients, and this individual had had "recurrent ENL", characterized by a chronic, turbulent clinical course which was not well controlled with medication. This provides intriguing insight into the mechanism of recurrent ENL, and suggests that the immunologic mechanisms involved may be a combination of the mechanisms operating in uncomplicated ENL and uncomplicated reversal reactions.

An abstract of these results (attached) has been submitted to the US-Japan Leprosy Panel, and a full manuscript is in preparation. Among the further studies to be conducted is the identification of cells producing IL-2 using another monoclonal antibody, and characterization of the phenotype of the IL-2-producers and the IL-2R+ cells.

Clinical studies of ENL. Detailed clinical records have been kept of study patients hospitalized with either Reversal Reactions or ENL (eg., Fig. 2). From these and the results noted above concerning an increase in IL-2R+ cells in the epidermis in a patient with "recurrent ENL", we have begun a review of our clinical data in order to clarify the differences in clinical signs, symptoms, and course of illness in

patients with typical ENL and "atypical" or "recurrent" ENL. These patients suffer more individually than patients with typical ENL, and they require much more hospital care and expense in their treatment. It may be that much of the long-term difficulties generally regarded as consequences of ENL are in fact seen primarily or exclusively in this group; identifying them by laboratory means so that they could be followed more closely, and intervention in complications initiated earlier, would accomplish one of the overall goals of this project. The studies we are conducting may also provide valuable information about the mechanisms responsible for these reactions, which might open up new opportunities for medical intervention or prevention.

Studies of Soluble substances in Blister Fluid. We have saved and frozen the fluid obtained from blisters after filtration to remove the cells, and have recently begun studies of soluble materials present in the blister. In collaboration with Dr. James Douglas, University of Hawaii, we have measured M. leprae-specific IgM (Table 2), and aliquots have been sent to Dr. David Wagner, Institute of Metabolic Disease, NIH, for assay of soluble IL-2R (Table 3).

Both of these studies are very preliminary, but they do indicate the feasibility of measuring these and other soluble immunologically active substances in blisters. Further possibilities to be explored in this regard include the presence of IL-2, gamma-interferon, immune complexes, and prostaglandin E-2.

Future Plans:

- 1) New patients will continue to be added to the study, as a means of increasing the basic patient population for long term follow up.
- 2) Follow-up studies will be continued, also.
- 3) As indicated in the discussion above, we expect to derive particularly important and useful information from studies of IL-2R+ and OKT-6+ cells. Additional markers being studied in Honolulu, for possible use in the field, include macrophage monoclonal antibodies and anti-IL-2 monoclonal antibody.
- 4) A second SCI proposal is now being drafted, proposing to use the results noted above to develop a more simplified method for clinical application of this information, using tissue fluid obtained from slit-skin smears. Since these smears are done routinely in leprosy hospitals worldwide, such a development would make additional diagnostic information available to all leprosy workers, not only those with access to larger hospital or research facilities.

INCREASED NUMBERS OF IL-2 RECEPTOR-BEARING CELLS IN THE EPIDERMIS
CHARACTERIZE LEPROSY REVERSAL REACTIONS

D. M. Scollard¹, S. Rongdaeng², V. Suriyanond², C. Theetrant²,
S. Makonksakayoon², R. Arnold, and T. Smith³ (1. Leprosy Atelier,
University of Hawaii, Honolulu, Hawaii; 2. Chiang Mai University, Chiang
Mai, Thailand; 3. McKean Rehabilitation Institute, Chiang Mai, Thailand.
Supported by USAID grant #PDC-5542-0-SF-5033-0).

"Reversal" (Type I) reactions in leprosy are sudden episodes of increased inflammatory activity in pre-existing lesions in the skin, nerves, and other sites. These are serious events, often medical emergencies, due to the extensive nerve damage that may occur in only a few days, leading to crippling deformity. These reactions have been defined almost entirely in clinical terms and are widely ascribed to "increases in cell-mediated immunity (CMI)", although very little immunologic evidence has been obtained to support this view.

Antigenic stimulation of T lymphocytes, as occurs in CMI reactions, results in the synthesis of interleukin-2 (IL-2) and the expression of IL-2 receptors (IL-2R) on T lymphocytes. To examine the hypothesis that reversal reactions are manifestations of CMI in leprosy, we have studied 19 biopsies from 18 patients, including 7 with clinical reversal reactions and 4 with ENL, to determine the micro-anatomic distribution and relative frequency of lymphocytes bearing IL-2R.

Patients were evaluated and classified according to diagnosis at McKean Rehabilitation Institute, Chiang Mai, Thailand. Diagnostic punch biopsies were obtained from active skin lesions as clinically indicated. Frozen sections of each biopsy were stained with a panel of monoclonal

antibodies including Leu 3_{a+b}, OKT-8, IL-2R, and OKT-6, using immunohistochemical single- and double-labelling techniques. The number of positively stained cells in both the dermis and epidermis was determined; results for the epidermis are expressed as the number of positive cells per 100 basal nuclei (BN).

Biopsies of patients with uncomplicated leprosy of all types, and with ENL, had 6 IL-2R+ cells/100 BN or less. In reversal reactions, however, 16-26 IL-2R+ cells / 100 BN were observed, a 3-fold or greater increase. In one patient biopsied sequentially early and late in the course of a reversal reaction, the number fell from 25 to 9 IL-2R+ cells, reminiscent of the decline we described previously in circulating soluble IL-2R levels in serum during the resolution of reversal reactions (Tung et al, 1985). One patient with clinical "downgrading reaction", and another with reversal reaction who had received a course of corticosteroids until shortly before biopsy, also showed low numbers of IL-2R+ cells in the epidermis. In addition, one patient with chronic, recurrent ENL had 12.7 IL-2R+ cells/ 100 BN, intermediate between the low values seen in uncomplicated disease and ENL, and the high values of reversal reactions.

The majority of IL-2R+ cells were observed to be in close contact with OKT-6+ cells, probably Langerhans cells. Finally, although some variation was seen in the number of IL-2R+ cells in the dermal infiltrates, they were much less apparent than the results in the epidermis.

These findings provide support for the hypothesis that reversal reactions are manifestations of enhanced CMI in leprosy, and augment the previous, similar findings on soluble IL-2R levels in serum in reversal reactions. The microanatomic distribution of these cells may also provide clues for the further understanding of reversal reactions.

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TABLE 1

EPIDERMAL IL-2R+ CELLS IN LEPROSY BIOPSIES

EX	NOKEAN	CLINICAL	BIOPSY	REACTION	IL-2R+	BASAL	IL-2R/BC
H	H	DX.	DX.		CELLS	CELLS	
30	10027	BT			0	300.00	0.0
16	9975	LL	LL		4	206.00	1.9
31	10028	BT	BT		10	324.00	3.0
1	9947	LL	LL		5	176.00	3.6
10	9961	LL	LL		19	460.00	4.1
12	9968	BL	BL		8	192.00	4.1
6	9489	LL	LL		31	528.00	5.8
9	9962	BT	BT		18	310.00	5.6
35	10041	BL		RR	7	229.00	3.9
3	9920	BB	BB	RR	26	298.00	8.7
28	10017	BB	BB	RR	19	201.00	9.4
8	9958	BL	BT	RR	51	325.00	15.6
11	9966	BL	BT	RR	87	385.00	22.5
27	10017	BB	BB	RR	65	334.00	25.4
7	9957	BB	BB	RR	65	326.00	26.0
14	9775	LL	LL	ENL	3	199.00	1.5
4	9919	LL	LL	ENL	11	358.00	3.0
36	10045	BL	BL	ENL	13	285.00	4.5
32	10015	LL	LL	ENL	51	399.00	12.7

Table 2

<u>Pt. #</u>	<u>ENL</u>		<u>Pt.#</u>	<u>RR</u>		<u>No. Rxn.</u>	
	<u>Phase</u>	<u>BI</u> <u>+/-</u>		<u>Phase</u>	<u>+/-</u>	<u>Pt.#</u>	<u>+/-</u>
14/3	3	-	29/3	5	+(24-96)	21/2	+
14/4	2	-	30/2	5	-	32/1	-
15/4	3	+(96 hr)	30/3	5	-	39/1	-
26/2	3	-	36/1	3	+(24-96)	41/1	-
26/3	4	-	36/2	4	+(30-96)	42/1	+(incre.30-96)
28/3	5	+	36/3	3	+(30-96)	43/1	+(96)
33/1	3	+(96 hr)	40/1	3	-	44/1	0
33/2	5	-				45/1	+(48)
35/1	3	+(48-96)				46/1	-
37/1	3	-				47/1	+(30-72)
38/1	3	+(24-72)				48/1	+(48-96)
38/2	3	-				49/1	+(18-96)

Positive in 5/12 episodes of ENL;

4/8 max ENL

Positive in 4/7 episodes of RR;

2/3 during Max. Rxn.

TABLE 3

Related biopsies studied for IL-2R in pts.

Pt. #	Biopsy #	Class	Reaction	Epic IL-2R (% per 100 EN)	(ENL) 37 down gr.		Phase
					(Units per ml) IL-25	Time	
(Ex. 14) 15, with NL	19, RR	32. No. R	33, ENL recur.	36, RR.	4996	(96 hr)	5
33	32	LL	ENL	12.7	2460	(96 hr)	3
29	27	BB	RR	25.4	5312	(24 hr)	3
					1730	(48 hr)	3
					10336	(96 hr)	1
36	35	BL	RR	3.9	2856	(24 hr)	3
					4608	(72 hr)	3
					3430	(96 hr)	3
32	31	BT	None	3.0	0	(49-72)	1
37	36	BL	ENL	4.5	0	(24-96)	3

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PROGRESS REPORT

USAID/SCF Office of Science Advisor Number 5.141

Title: "Development of an Immunoperoxidase test for Early Diagnosis of Acute Reactional States in Leprosy"

Project Period: July 1, 1986 - Dec. 30, 1986. Third reporting period.

Investigators:

David M. Scofield, M.D., Ph.D., Department of Pathology, University of Hawaii School of Medicine;

Choti Theetranont, M.D., Department of Pathology, Chiang Mai University, Chiang Mai, Thailand;

Vinai Suriyanoud, M.D., Department of Medicine, Chiang Mai University, Chiang Mai, Thailand;

Sarit Makonkornkeyoon, Ph.D., Department of Clinical Immunology, Chiang Mai University, Chiang Mai, Thailand;

Samreung Rangdaeng, M.D., Department of Pathology, Chiang Mai University, Chiang Mai, Thailand.

Rec'd in SCI: FEB 27 1987

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Publications resulting from this work:

Scollard, D.M., Rangdaeng, S., and Kenney, R.T. (1985) "Evolution of cutaneous immune responses in tuberculosis and leprosy" Lab. Invest. 52: 60.

Kenney, R.T., Rangdaeng, S., and Scollard, D.M. "Cellular Kinetics of the Human Immune Response to PPD Using Suction-Induced Skin Blisters: An Immunocytochemical Study" J. Immunol. Methods, Jan 1987. In press.

Scollard, D.M., Rangdaeng, S., and Kenney, R.T. 1985 "Kinetic patterns of cellular response to repeated tuberculin testing in healthy responders and in subclinical tuberculosis; Correlates to the 'Booster effect'" (Submitted to Clin. Immunol. Immunopath.)

Tung, K.S.E., Nelson, K., Rubin, L., Wagner, D., Umland, E., Schauf, V., Scollard, D., Vithayasai, P., Vithayasai, V., Worobec, S., Smith, T., and Suriyanond, V. 1985 "Serum soluble interleukin-2 receptor levels in leprosy patients" Presented at the US-Japan Leprosy Panel, Aug, 1985, Bethesda, Md (Inf. Immun., in press)

Scollard, D.M., Rangdaeng, S., Suriyanond, V., Theetranont, C., Makonkawkeyoon, S., Arnold, R., and Smith, T. "Increased Numbers of IL-2 Receptor-Bearing Cells in the Epidermis Characterize Leprosy Reversal Reactions" Lab. Invest., Feb., 1987. In press.

Manuscripts in preparation:

Rangdaeng, S., Scollard, D.M., Suriyanond, V., Makonkawkeyoon, S., and Theetranont, C. "Mononuclear cell subsets from uncomplicated cutaneous lesions of leprosy in man: An immunocytochemical study using suction-induced skin blisters".

Scollard, D.M., Tung, K.S.K., Rubin, L., Wagner, D., et al "Soluble Interleukin-2 receptor levels in fluid from suction-induced blisters of cutaneous leprosy lesions"

Suriyanond, V., Scollard, D.M., Rangdaeng, S., et al "The Clinical Course of Simple and Recurrent Erythema Nodosum Leprosum: A Clinical Study"

Scollard, D.M., Suriyanond, V., Chang, P.H.C., Kimura, L., Smith, T., and Oishi, N. "T-helper and T-suppressor Cell Subsets in Human Leprosy Lesions Studied in vivo"

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Project Objectives:

Unchanged;

1. To determine, in skin lesions of leprosy patients, changes in the distribution and/or ratio of lymphocyte and monocyte subpopulations which accompany acute reactions in leprosy, using monoclonal antibodies.
2. To develop a non-invasive "skin window" method of monitoring lymphocyte and monocyte changes that will enable early identification of patients with a high risk of impending reaction.

Progress of Project Activities

1. There have been no changes in staff during this reporting period. Work has proceeded smoothly, with continued follow up of patients studied previously, and intake of additional new patients. During most of this period, Dr. Trevor Smith was away on a home leave, which did cut down somewhat on clinical capabilities, but since the project and procedures are now well established, this had no serious deleterious effect.

Our project staff have been involved in starting up the joint work to be done under the auspices of the WHO-funded Tropical Disease Research Unit of RHES. This will provide one means for the continuation of the present work after the grant from USAID has expired. In addition, a pre-proposal has been submitted to the AID/SCI program in order to pursue some new possibilities of more simplified clinical diagnostic techniques.

2. At the time of this writing 118 active patients and 36 inactive control patients have been studied, many of them on multiple occasions (one patient has now been studied eight times, before, during, and after multiple reactions). This represents an increase of 30 patients in the six months elapsed since the last report; the majority of these have been new patients without reactions, who are now being followed.

The panel of antibodies currently being used in Chiang Mai is:

Leu-4	all T cells
Leu-3	T-helper cells
OKT-8	T-suppressor cells
IL-2R	Activated cells bearing the IL-2 receptor
OKT-6	Langerhans cells.

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A major technical step is now in progress, involving multiple-parameter analysis of the blister exudate cells using more than one monoclonal antibody, labelling the cells in Thailand, and examining a portion of the sample in Thailand as before, while another portion is sent to Hawaii for flow cytometry. This will enable us to obtain substantially more detailed information to guide further efforts using our standard methods.

Results

Findings of greatest interest continue to be related to the numbers and distribution of IL-2-receptor bearing cells, the clinical features of ENL (which may be correlated with the findings on IL-2R), and the measurement of antibody and IL-2R in the blister fluid. In addition, we are now able to identify not only the types of cell present, but also the functional subtype using the multiple-labelling methods and flow cytometry described above.

IL-2 Receptors. Lymphocytes which have been stimulated by specific antigen, and which will proliferate as a result, also produce the immunoregulatory substance designated "interleukin 2" (IL-2). For IL-2 to stimulate other cells, and thus amplify the immune response, other lymphocytes must express increased amounts of IL-2 receptors (IL-2R) in their surface membranes. Identification of such IL-2R+ cells, therefore, is a measure of immunologic stimulation which is most directly related to initiation of cell-mediated immunity.

As noted in the last report, we have observed that in skin biopsies from patients with reversal reactions, the number of IL-2R+ cells is increased. This increase was not seen in a patient with a "downgrading reaction", nor in a patient who had been treated recently with corticosteroids. Moreover, the number of IL-2R+ cells in the epidermis declined with treatment in one patient who had been biopsied just at the outset of his reaction, and again three weeks into his treatment. It now appears, however, that not all biopsies of reversal reactions show these changes, and it is not yet clear whether this is related to the timing of the biopsy (related to the onset of reaction), or if there are in fact different populations of patients in reaction, whose skin lesions display different immunopathologic features.

Of special interest is that the increased number of IL-2R+ cells is observed in the epidermis, rather than in the dermis where the leprosy bacilli and associated inflammatory infiltrate are located. We believe that this may be related to increased functional activity on the part of Langerhans cells, the principal antigen-presenting cells in the skin, which are also located in the epidermis. We are therefore focussing specifically on the identification and enumeration of Langerhans cells in our biopsies and blister fluids.

These results are being presented to the meeting of the US-Canadian Division of the International Academy of Pathology, in Chicago in March, and a full manuscript is in preparation.

Multiple Fluorochrome Labelling and Flow Cytofluorometric Analysis.

Among the further studies to be conducted is the identification of cells producing IL-2 using another monoclonal antibody, and characterization of the phenotype of the IL-2-producers and the IL-2R+ cells. The initial work on this will be done using flow cytometry to identify the most promising cells and antibodies, and these results will determine the direction of subsequent immunostaining in Chiang Mai.

Preliminary results of studies in Hawaii to adapt this technique to the examination of blister exudate cells are presented in Table 1 and Figures 1 & 2. We are using the human skin test response to PPD as a reasonably well-defined immunologic stimulus, as a means of establishing reference points for interpretation of data on leprosy. Several tuberculosis patients and healthy volunteers have been studied in Hawaii, as shown in Table 1. From these preliminary results, it appears that in PPD+ individuals in both groups, T-suppressor cells (OKT8+) outnumber T-helper cells (LEU3+) during the first 48 hours of the response to PPD, but that this ratio reverses between 48 and 72 hours. In PPD-negative individuals, T-helper cells are often the predominant group throughout the response.

Recent advances in cellular immunology have revealed that these two subsets are themselves heterogeneous, and that co-expression of other cell markers is a useful means of differentiating the functional subsets within each of these groups. Figures 1 and 2 demonstrate how this is done by double-labelling these cells with Leu2/Leu15 (Fig. 1) and Leu3/Leu8 (Fig. 2). These results are quite promising, but much more work must be done before we may draw any conclusions.

Clinical studies of ENL. The clinical data concerning the course of ENL continues to be analyzed. Because of our decreased clinical capabilities due to Dr. Smith's leave, this work has not progressed a great deal since the last report, although additional patients and follow-up have been added to our database.

Studies of Soluble substances in Blister Fluid. As noted in the last report, we have saved and frozen the fluid obtained from blisters after filtration to remove the cells, and have recently begun studies of soluble materials present in the blister. Since our preliminary studies of these blister fluids were quite promising (as noted in the last report), we have now undertaken major screening of these fluids for anti-M. leprae antibodies (in collaboration with Dr. James Douglas, University of Hawaii) and assaying for soluble IL-2R (with Dr. David Wagner, Institute of Metabolic Disease, NIH).

The majority of the laboratory work on IL-2R has been completed (approximately 600 samples), and data are now being analyzed. Work on the antibody levels has just begun. Further possibilities to be explored in this regard include the quantitation of IL-2, gamma-interferon, immune complexes, and prostaglandin E-2.

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Future Plans:

- 1) New patients will continue to be added to the study, as a means of increasing the basic patient population for long term follow up.
- 2) Follow-up studies will be continued, also.
- 3) The major technical step to be taken in the next few months concerns labelling of cells for flow cytofluorometry, permitting functional lymphocyte subsets to be identified.
- 4) A second SCI pre-proposal has been submitted, proposing to use the results noted above to develop a more simplified method for clinical application of this information, using tissue fluid obtained from slit-skin smears. Since these smears are done routinely in leprosy hospitals worldwide, such a development would make additional diagnostic information available to all leprosy workers, not only those with access to larger hospital or research facilities.

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LYMPHOCYTE SUBSETS (% OF MONONUCLEAR CELLS)

PATIENT NO.	TYPE	PPD	HR	TOTAL COUNT	% MNC	% LEU4+	% LEU5+	% TS+	% IL2R+	% DR51+
PH05	TUB	20.0	30	3226	55	47	29	34	27	0
PH05	TUB	20.0	48	3517	93	25	17	26	17	0
PH05	TUB	20.0	72	12607	99	21	22	10	8	0
PH08	TUB	0.0	30	20996	92	34	18	13	51	0
PH08	TUB	0.0	48	4133	82	38	32	29	57	0
PH08	TUB	0.0	72	21267	85	46	23	14	22	0
PH10	HEA	0.0	24	1894	61	22	14	17	0	0
PH10	HEA	0.0	30	6497	82	11	5	10	0	0
PH10	HEA	0.0	48	634	97	12	17	6	0	0
PH10	HEA	0.0	72	10676	96	45	27	16	0	0
PH11	HEA	0.0	24	12535	95	18	12	5	0	0
PH11	HEA	0.0	30	3560	96	20	8	8	0	0
PH11	HEA	0.0	48	6380	93	23	18	19	0	0
PH11	HEA	0.0	72	23141	93	35	35	8	0	0
PH12	HEA	6.5	24	164344	36	25	4	4	0	0
PH12	HEA	6.5	48	13546	72	26	5	4	0	0
PH12	HEA	6.5	72	57507	80	41	28	17	0	0
PH12	HEA	6.5	96	40951	89	47	26	16	0	0
PH13	HEA	0.0	18	18951	36	9	2	2	15	0
PH13	HEA	0.0	24	17989	61	26	1	7	6	0
PH13	HEA	0.0	48	19366	84	35	22	10	0	0
PH13	HEA	0.0	72	25322	74	41	41	15	0	0
PH14	HEA	0.0	30	8411	72	14	6	17	19	0
PH14	HEA	0.0	48	3643	63	31	8	19	9	0
PH14	HEA	0.0	72	25541	75	20	17	12	13	0
PH14	HEA	0.0	96	10765	86	25	10	6	21	0
PH15	HEA	0.0	30	41221	96	16	34	13	7	0
PH15	HEA	0.0	48	8826	89	24	24	13	3	0
PH15	HEA	0.0	72	21132	97	38	30	14	2	0
PH15	HEA	0.0	96	35537	90	42	37	10	0	0
PH16	HEA	9.5	30	27958	36	14	11	11	6	0
PH16	HEA	9.5	48	44287	43	21	9	10	3	0
PH16	HEA	9.5	72	15254	30	24	17	11	0	0
PH18	TUB	15.0	30	39484	89	26	16	27	2	0
PH18	TUB	15.0	48	65743	73	33	31	17	0	0
PH18	TUB	15.0	72	76204	91	45	11	33	4	0
PH18	TUB	15.0	96	55732	67	29	16	21	3	0

Figure 1. Two-Color Flow Cytometric Analysis of Blister Exudate Cells in PPD Response in vivo: Suppressor cells.

The volunteer was an inpatient at Leahi Hospital, with active tuberculosis and a positive PPD test. Results are shown for (A) 24 hours and (B) 48 hours after PPD.

Cells from one blister were labelled with PE-Leu2 plus FITC-Leu15, producing orange and green fluorescence, respectively. Data here are presented as intensity of orange fluorescence (x axis) against intensity of green fluorescence (y axis); the number of points in each quadrant is correlated with the number of cells present. In this presentation, cells represented are:

<u>Quadrant</u>	<u>Fluorescence</u>	<u>Cell type</u>
1	Green only	Leu2-/15+; suppressor cells.
2	Orange & Green	Leu2+/15+; Functional suppressor cells.
3	None	Not suppressor cells (other T, B cells)
4	Orange only	Leu2+/15-; Suppressor and cytotoxic cells.

The percentage of T-suppressor cells (ie, all orange fluorescence) increased from 5% at 24 hours to 20% at 48 hours, but the percentage of double-labelled cells (ie, functional suppressor cells, quadrant 2) dropped from 1% to 0% over the same interval.

Figure 2. Two-Color Flow Cytometric Analysis of Blister Exudate Cells in PPD Response in vivo: Helper Cells.

Data here are from the same patient and samples as in Fig. 1, but these aliquots of cells were labelled with PE-Leu3 (orange), and FITC-Leu8 (green). Results also show cell profiles at 24 hr. (A) and 48 hr. (B) after PPD.

Data are presented as in Fig. 1, and designations for the different quadrants are as follows:

<u>Quadrant</u>	<u>Fluorescence</u>	<u>Cell type</u>
1	Green only	Leu3-/8+; Helper cells; mixed functions
2	Orange & Green	Leu3+/8+; Functional helper cells (for B-cell function)
3	None	Not helper cells (other T, B cells, etc.)
4	Orange only	Leu3+/8- Helper cells; "suppressor-inducer" cells

The number of helper cells (ie all orange fluorescence) increased from 0.5% at 24 hr. to 7% at 48 hours, and the number of "suppressor-inducer cells (ie., those which stimulate T-suppressor cells) appears to have increased proportionally.

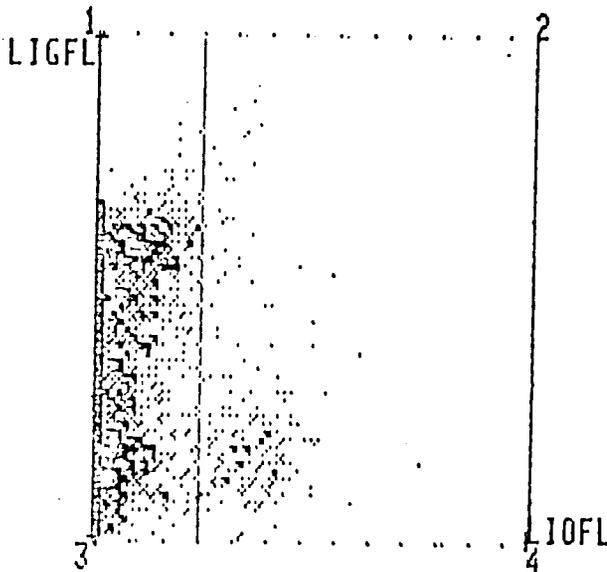
Fig 1.A

POSITION OF

VERTICAL
X= 15

HORIZONTAL
Y= 20

LOWEST LEVEL
1



VERTICAL
CURSOR

HORIZONTAL
CURSOR

DATA TO
PRINTER

QUAD	PERCENT	PEAK	POS	PEAK HT	AREA
1	27.55	0,	35	31	883
2	1.12	18,	40	2	36
3	66.90	0,	5	226	2144
4	4.43	19,	7	4	142

READY

RETURN TO
CONTOUR

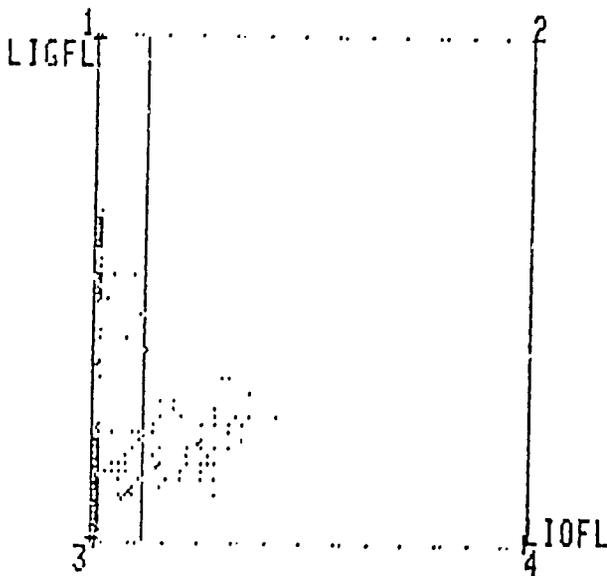
Fig 1.B

POSITION OF

VERTICAL
X= 7

HORIZONTAL
Y= 24

LOWEST LEVEL
1



VERTICAL
CURSOR

HORIZONTAL
CURSOR

DATA TO
PRINTER

QUAD	PERCENT	PEAK	POS	PEAK HT	AREA
1	18.26	0,	38	6	42
2	0.	8,	25	0	0
3	62.17	0,	4	19	143
4	19.57	9,	9	2	45

READY

RETURN TO
CONTOUR

21

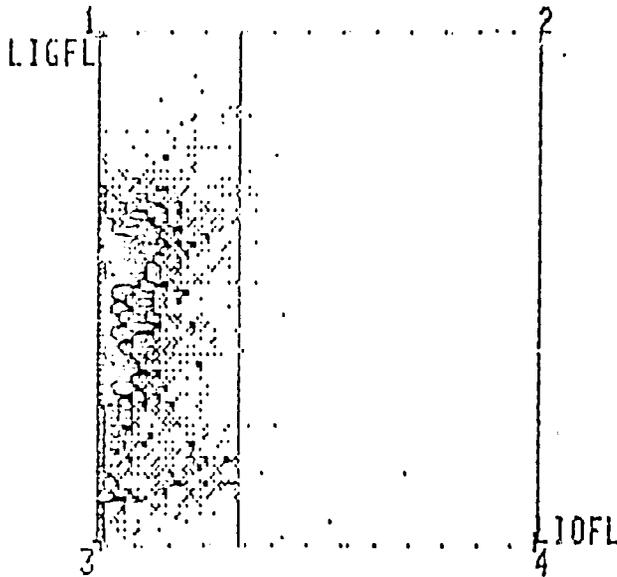
Fig 2 A.

POSITION OF

VERTICAL
X= 20

HORIZONTAL
Y= 24

LOWEST LEVEL



VERTICAL
CURSOR

HORIZONTAL
CURSOR

DATA TO
PRINTER

QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	38.47	0, 25	46	1434
2	0.27	21, 43	1	10
3	61.11	0, 5	221	2278
4	0.16	21, 14	1	6

READY

TOTAL COUNT - 4300

RETURN TO
CONTOUR

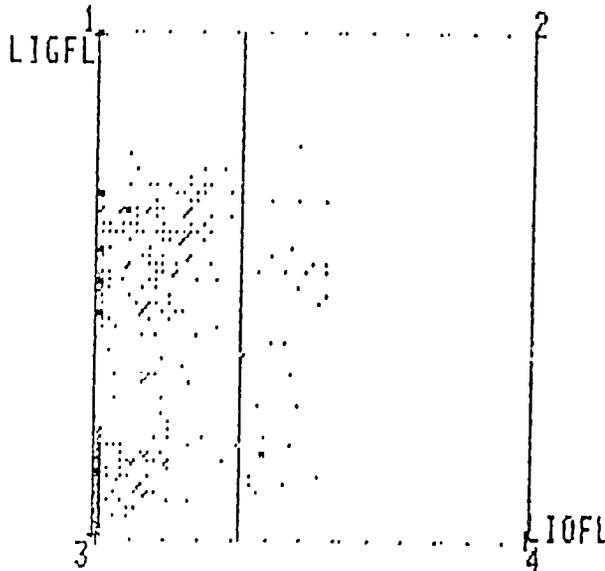
2. B.

POSITION OF

VERTICAL
X= 21

HORIZONTAL
Y= 23

LOWEST LEVEL



VERTICAL
CURSOR

HORIZONTAL
CURSOR

DATA TO
PRINTER

QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	32.33	0, 36	4	139
2	3.95	23, 33	1	17
3	60.70	0, 5	36	261
4	3.02	24, 10	3	13

READY

RETURN TO
CONTOUR

W