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5.148

FINAL PROGRESS REPORT

Project Title:

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:

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Project Leader

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Project Profile

Country:	Thailand
Grant Number:	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, MD
Organization:	Department of Pathology Faculty of Medicine Chiang Mai University
Co-Investigators:	David M. Scollard, MD, PhD Kamthorn Thamprasert, MD Lertlakana Bhoopat, MD Vinai Suriyanon, MD Samreung Rangdaeng, MD Sanit Makonkawkeyoon, PhD
Project Consultants:	(1) Trevor Smith, MD Medical Director McKean Rehabilitation Center (2) David Wagner, PhD David Nelson, PhD Immunophysiology Laboratory National Cancer Institute Bethesda, Maryland, USA

Authorized Officer:	Choti Theetranont, MD
Total Project Budget:	Bt 2,899,800
Project Duration:	1 April 1985 through 30 November 1987
Reporting Period:	Final
Budget Allocation for this Period:	See attached financial report (ANNEX A) for the period ending 30 November 1987

Contents

1. Background and Introduction:

Through the period ending 31 August 1987, 164 patients were enrolled in this study. (Additional patients are being studied using a very similar protocol and follow up is continuing on this original cohort under a grant from the WHO Special Programme for Research and Training in Tropical Diseases, discussed below). Of these, 31 were inactive control patients, and 10 were patients tested with lepromin. The remaining 123 patients had active disease, with or without reactions.

2. Objectives:

2.1. To determine, in skin lesions of leprosy patients, changes in the distribution and/or ratio of lymphocyte and monocyte populations which accompany acute reactions in leprosy, using monoclonal antibodies.

2.2. 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.

3. Materials and Methods: See previous reports.

4. Results/Discussion/Tables:

Technical aspects of this project have been very successful. Our original methods of fixation and staining of cells on filters have been improved by slight modifications which have proved to be a relatively simple and reproducible method. The cell-free filtrate, composed of blister fluid and medium, has been a richer source of information than expected, largely because of advances in biotechnology enabling development of molecular assays of potent, soluble immuno-regulatory substances such as interleukin-2 and its receptors, gamma interferon, and so forth. When this project began, the only assays for such substances were complex bioassays requiring larger volumes of material than can be obtained from blisters. We therefore have been fortunate in having the active consultation and collaboration of Dr David Wagner and

Dr David Nelson, Immunophysiology Laboratory, National Cancer Institute, USA, who have helped us to take advantage of these developments. The general technical approach has been received with notable interest at scientific meetings where it has been presented (Scollard, 1987).

The response to our original publication (Kenny, Rangdaeng, Scollard, 1987) has been gratifying. Over 300 reprint requests have been received, the bulk of them from other developing countries, where the applicability of this method is appreciated.

Analysis of the data on cellular infiltrates within blisters has been slower and more difficult than expected, since great care must be taken to ensure acceptable quality staining for all cell counts included in the final results. Variability of staining occurs from day to day because of factors beyond technical control, and control cells are stained along with blister-derived cells to check and maintain quality. When unacceptable quality is observed, it is usually only one of the several antibodies in the panel that gives a poor result at any one time. Some of the parameters studied, however, are ratios, and these cannot be calculated for a given blister if one of the figures is omitted. The careful "weeding out" of such data is time-consuming; but having 3-4 blister samples from each patient ensures that reliable data is obtained from every patient.

4.1. Cellular Studies:

Data on patients with various types of active leprosy, without (or before) reactions, has been analyzed on 28 patients, presented in figures 1-4 attached. These are draft figures for a manuscript in preparation by Rangdaeng et al..

Total cell counts usually ranged from 10-80,000 and fell into three patterns. In LL and BL patients cellularity from 24-96 hours remained relatively constant at the low end of this range (Figures 1A, B). (The broken line in these figures indicates blisters in which 40% or more of the cells are polymorphs, indicating that the blister may have broken before aspiration. These data have been deleted from subsequent analyses). In several lepromatous patients - and in most of the BT ones- cellularity was lowest at 48 hours and rose to varying levels thereafter. Finally, in some BT patients, a reverse pattern was observed, with maximum cellularity at 48 hours (Figure 1C).

Since the total cellularity does not vary extensively, it is not yet clear whether these differences have biological importance. These findings are particularly interesting in the light of studies by other investigators, who have noted similar kinetics in the production of fibrin and fibrinogen in cell-mediated immune responses. Differences in time of maximum cellularity may therefore be influenced by the production of these materials in the dermis, varying the degree of "freedom" of monocytes and lymphocytes to enter the blister.

In the great majority of cases, over 70% of the cells in the blister exudate were mononuclear cells at all times studied (Figure 2A-C). This was not related to the type of leprosy. This is important because it establishes that the cells studied are those responsible for cell-mediated immunity; and this is true across the spectrum of the disease, so that direct comparisons of subsets can be made.

The ratio of T lymphocytes to monocytes (T:M ratios) among these mononuclear cells were also similar across the spectrum (Figure 3), with the exception that BT patients showed a significantly greater percentage of lymphocytes at 48 hours. It is not clear whether this is of biological significance, and it may also be related to the production of fibrin, et cetera, as noted above. This is currently under investigation.

Finally, the ratios of T-helper to T-suppressor cells (H:S ratios) are higher in BT patients at all times up to 96 hours (Figure 4). This is consistent with all other studies of T cell subsets in leprosy lesions. The observation that by 96 hours the ratios are similar to all types of leprosy may be related more to the biology of migration of cells into the blister than to the biology of leprosy. This too is under investigation.

In general, these studies have provided results consistent with those of prior studies of biopsies, but with greater precision. They also appear to demonstrate a kind of immunologic equilibrium existing in leprosy lesions of different types which are not in reaction. Preliminary results of these analyses of studies of patients with reversal reactions with ENL support our original hypothesis that predictable, reproducible differences from this baseline are seen in each type of reaction (Bhoopat et al., Thamprasert et al., 1987).

Biopsy studies have demonostrated increased numbers of IL-2R-bearing cells in the epidermis in reversal reactions (Scollard et al., 1987). The implications of this finding are considerable, and the nature of these cells is being investigated.

4.2. Studies of Soluable Mediators in Blister Fluid:

The preceding discussion of cellular results has been somewhat detailed, since the manuscripts are still in preparation. The data concerning soluable IL-2R will be outlined briefly, since manuscripts detailed the results are attached.

4.3. IL-2 Receptors:

Lymphocytes which have been stimulated by specific antigen and 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 increaed 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. Stimulated cells also release IL-2 receptor, also designated as "Tac peptide", a marker increasingly recognized as an important indicator of enhanced immunologic activity.

4.3.1. Tac peptide in tuberculin tests:

In defined responses to tuberculin (PPD) skin tests, the maximum levels of Tac peptide are directly correlated with induration, the classical measurement of delayed hypersensitivity in man (Scollard, Wagner, and Nelson, 1988). This fundamental observation is important for our study of leprosy for several reasons:

- It demonstrates the correct correlation between a mycobacterial antigenic stimulus and one of the markers readily quantitated in this blister system.

- It demonstrates the temporal relationship between such stimulation and response in vivo.

- The method can be readily extended to similar testing with M. leprae antigens. This is currently being done here in Chiang Mai.

- It provides valuable reference information for results in leprosy lesions: in these lesions, the investigator has only a crude knowledge of and no control over the amount of antigen (M.leprae) in the skin according to the length of time it has been there.

4.3.2. Tac peptide in leprosy:

With few exceptions, we have found that Tac peptide levels in active patients are similar to those in inactive, cured controls. Moreover, the levels are similar across the spectrum from LL to BT. This is further evidence of the "steady state" in immunologic activity within active skin lesions.

In contrast, 50% of the patients with reversal reactions in this study had highly elevated levels of peptide in their skin lesions. The lesion in reversal reaction thus represents a deviation from "steady state", and through studies of these patients before, during, and after reactions we will learn much more about the immunologic responsiveness in both the reaction and the underlying condition. The results suggest also that elevations of Tac peptide may be related to the severity of the reaction or its duration, or both; and this is an important consideration in its use as a diagnostic or prognostic tool.

4.4. Antibodies:

In previous reports we have alluded to the finding of increased levels of anti-M. leprae antibody in blisters in ENL. Additional samples are now being assayed and the results suggest that such increases may have diagnostic and/or prognostic value with respect to ENL in lepromatous patients.

5. Summary and Conclusions:

5.1. In addition to the scientific contributions mentioned above, this project has made an important material contribution to the Department of Pathology, Faculty of Medicine, Chiang Mai University, and has promoted and facilitated the establishment of other immunological techniques for diagnostic use at the hospital.

Furthermore, the project has played an important role in the development of the Tropical Diseases Research Unit (TDRU) at the Research Institute for Health Sciences at Chiang Mai University. The establishment of the TDRU with support from the WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR) has enabled several physicians from the University to obtain special training abroad and has increased the profile of research on leprosy in Northern Thailand.

This project has also provided the means to follow up the initial cohort of patients enrolled in this longitudinal study beyond the two-year limitation of the USAID grant.

5.2. Suction-induced blisters have proved to be a simple, reliable method for the observation of intracutaneous immunologic events in leprosy lesions. Both cellular changes and soluble immunologic mediators have been examined with this method. Changes in both cellualr composition and levels of soluable mediators have been observed which correlate with with clinical changes in disease status, that is, from active "steady state" lesions to reversal reactions or ENL. The methods have been fully suitable for field conditions.

We have proposed to extend these observations to the examination of "slit-skin smears", a crude cutaneous sampling method used to determine the numbers of leprosy bacilli in the skin in all leprosy programs around the world. Adaptation of the results and methods, from those of the blister technique to the much smaller samples from skin smears, is feasible. Such adaptation would make the diagnostic and prognostic potential in these studies feasbile in leprosy control programs worldwide, most of which do not enjoy the proximity to a university or the congenial collaborative relationship which exists between the McKean Rehabilitation Center and Chiang Mai University.

6. References:

6.1. 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 responses to PPD using suction-induced skin blisters: An immunocytochemical study" (1987). J. Immunol. Methods 97: 101-110.

Bhoopat, L., Thamprasert, K., Suriyanon, V., Smith, T., Scollard, D., and Theetranont, C. "Evolution of in situ immune responses in clinical subgroups of Erythema Nodosum Leprosum reactions of leprosy: an immunoperoxidase study using suction-induced blisters" J Clinical Immunology and Immunopathology (Submitted for publication).

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 Suriyanon, V. (1987) "Serum soluable interleukin-2 receptor levels in leprosy patients" Clin. Exp. Immunol. 69: 10-15.

Scollard, D.M., Rangdaeng, S., Suriyanon, V., Theetranont, C., Makonkawkeyoon, S., Arnold, R., and Smith, T. (1987) "Increased numbers of IL-2 receptor-bearing cells in the epidermis characterize leprosy reversal reactions" Lab. Invest. 56: 70A.

Scollard, D.M., Wagner, D.K., and Nelson, D.L. (1988) "Release of soluable interleukin-2 receptors (Tac peptides) in vivo during human immune responses to tuberculin". Clinical Immunology and Immunopathology. (in press)

Scollard, D.M. (1988) "A review of immunopathologic studies of human leprosy lesions in situ. Hawaii Medical Journal, Special Issue on Hansen's Disease (in press).

Scollard, D.M., Suriyanon, V., Wagner, D.K., Smith T., Thamprasert, K., Nelson, D.L., and Theetranont, C. "Increased levels of intracutaneous soluable interleukin-2 receptors (Tac Peptides) in leprosy reversal reactions" Infection and Immunity (submitted for publication).

6.2. Manuscripts in Preparation:

Rangdaeng, S., Scollard, D.M., Suriyanon, 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".

Suriyanon, V., Scollard, D.M., Rangdaeng, S., et al.. "The clinical course of simple and recurrent Erythema Nodosum Leprosum: A clinical study".

Scollard, D.M., Suriyanon, 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".

7. Annexes/Tables:

7.1. Annex A: Financial Report for the Period through 30 November 1987.

7.2. Annex B: Tables 1A, 1B, 1C

13 January 1988 (Final Draft)

Annex A

DEVELOPMENT OF AN IMMUNOPEROXIDASE TEST FOR EARLY DIAGNOSIS

OF ACUTE REACTION STATES IN LEPROSY

GRANT NO 936-5542-G-00-5044-00

Status of Funds for the Period Ending 30 November 1987

	(1)	(2)	(3)	(4)
CATEGORY	BUDGET	FUNDS RECEIVED TO DATE	EXPENDITURES THIS PERIOD	TOTAL EXPENDITURES/ ACCRUED EXPENSES
1. Personnel	783,000	842,645.20	40,542.00	815,074.80
2. Consultants	243,000	243,000.00	6,700.00	253,075.00
3. Equipment	810,000	792,634.20	nil	707,739.36
4. Supplies	523,800	507,490.70	5,204.50	451,885.51
5. Travel	337,500	248,178.33	nil	292,460.32
6. Patient Costs	54,000	58,198.00	2,686.00	98,890.50
7. Shipping	121,500	107,505.40	5,000.00	150,191.22
8. Communications	27,000	35,762.52	829.50	33,437.35
TOTALS	2,899,800	2,835,414.35	60,962.00	2,802,754.06

Chiang Mai
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15 January 1987

11/24/87

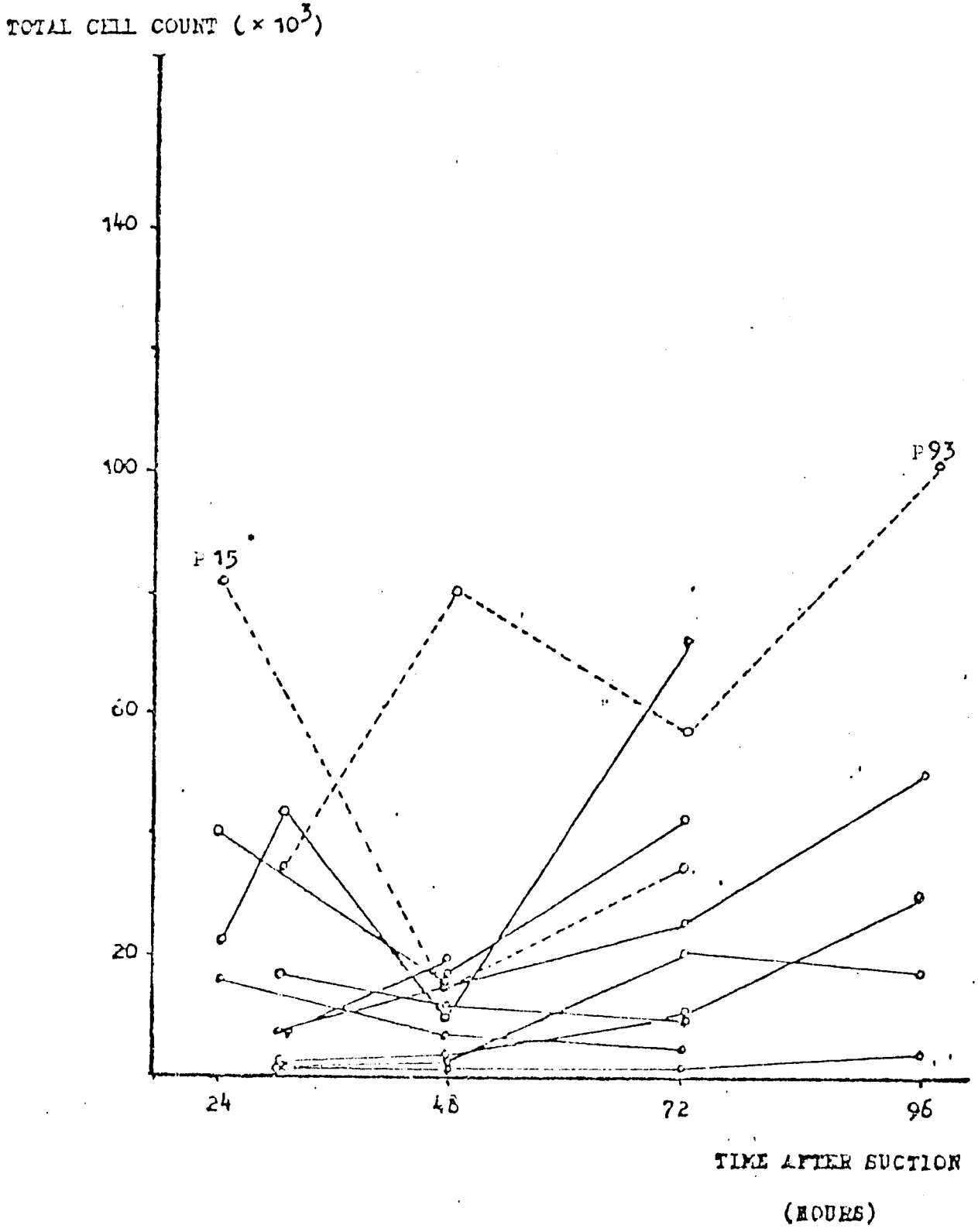
LEPROSY PATIENTS STUDIED: CLINICAL DATA AND BLISTER VOLUMES

PT #	AGE	SEX	TYPE	STATUS	24 HR VOL.	30 HR VOL.	48 HR VOL.	72 HR VOL.	96 HR VOL.
15	38	M	LL	ACTIVE	50		50	40	
21	18	M	LL	ACTIVE		30	50		
22	32	M	LL	ACTIVE	100		70	100	
28	28	M	LL	ACTIVE	50	50	40	50	
32	34	M	LL	ACTIVE	30		40	70	
42	69	M	LL	ACTIVE		70	50	60	30
43	59	M	LL	ACTIVE		50		70	80
62	33	M	LL	ACTIVE		30	30	50	50
67	40	F	LL	ACTIVE		50	50	30	50
86	30	M	LL	ACTIVE		60	100	70	80
93	39	M	LL	ACTIVE		50	50	60	80
11	28	M	BL	ACTIVE	70		50	50	
17	23	M	BL	ACTIVE	100		30	40	
24	26	M	BL	ACTIVE	130		80	130	70
27	71	M	BL	ACTIVE	80		60	130	140
39	55	M	BL	ACTIVE	30		40	50	50
44	21	M	BL	ACTIVE		60	110	100	100
51	44	M	BL	ACTIVE		[illegible]	120	110	60
53	54	M	BL	ACTIVE	50		50	30	60
58	55	M	BL	ACTIVE		50	80	80	90
82	47	M	BL	ACTIVE		70	60		
41	52	F	BB	ACTIVE	40		60	50	40
46	35	M	BB	ACTIVE		30	30	60	
45	28	M	BT	ACTIVE		50	60	50	60
48	47	M	BT	ACTIVE		30	70	90	90
61	53	M	BT	ACTIVE		40	30		50
72	16	M	BT	ACTIVE		110	90	110	
87	31	M	BT	ACTIVE		70	50	80	50

FIGURE 1A

TOTAL CELL COUNTS

ACTIVE LL LEPROSY $\bar{B}$ REACTIONS

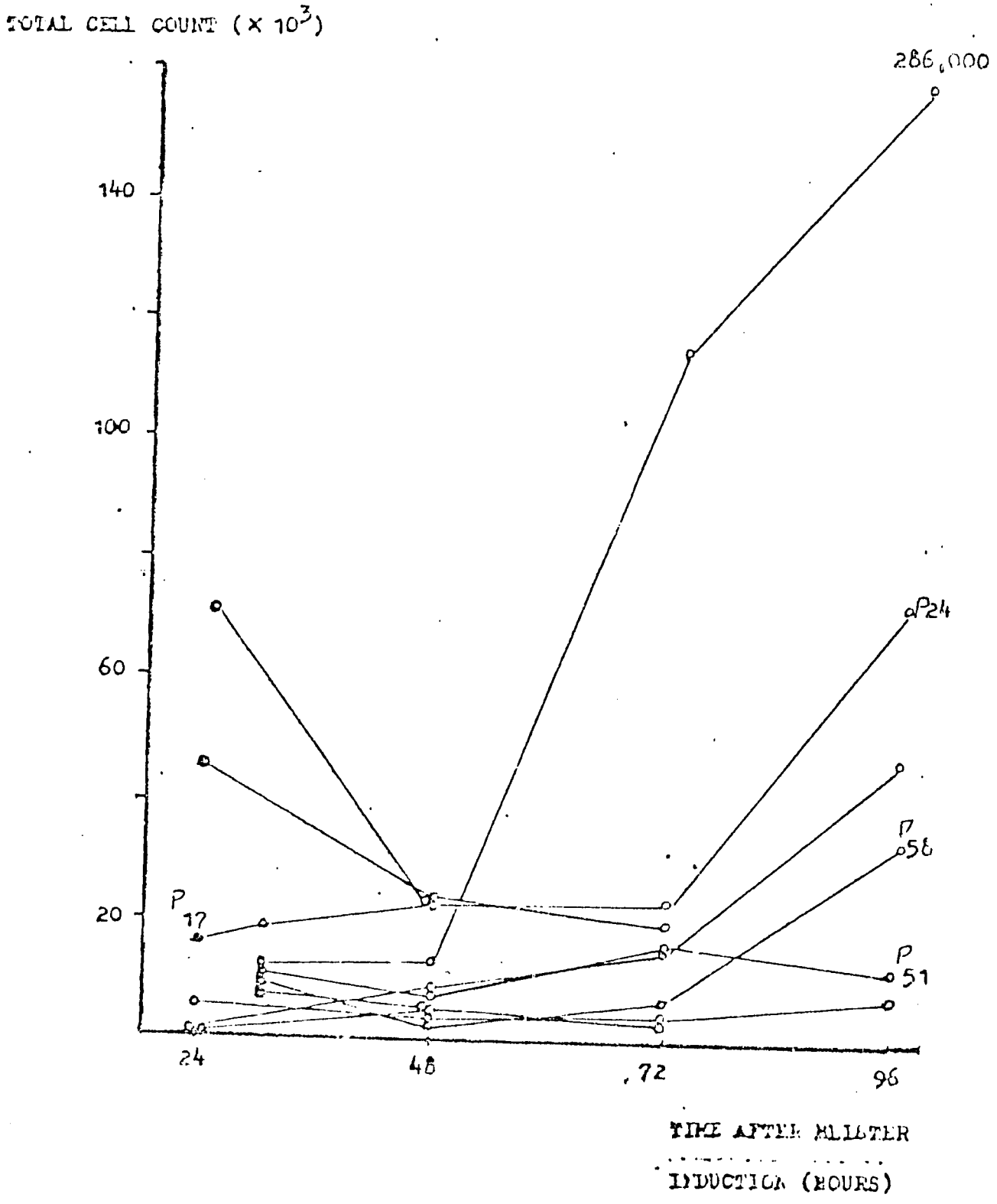


*Indicates patient number.

FIGURE 1B

TOTAL CELL COUNTS

ACTIVE EL $\bar{L}$ REACTIONS

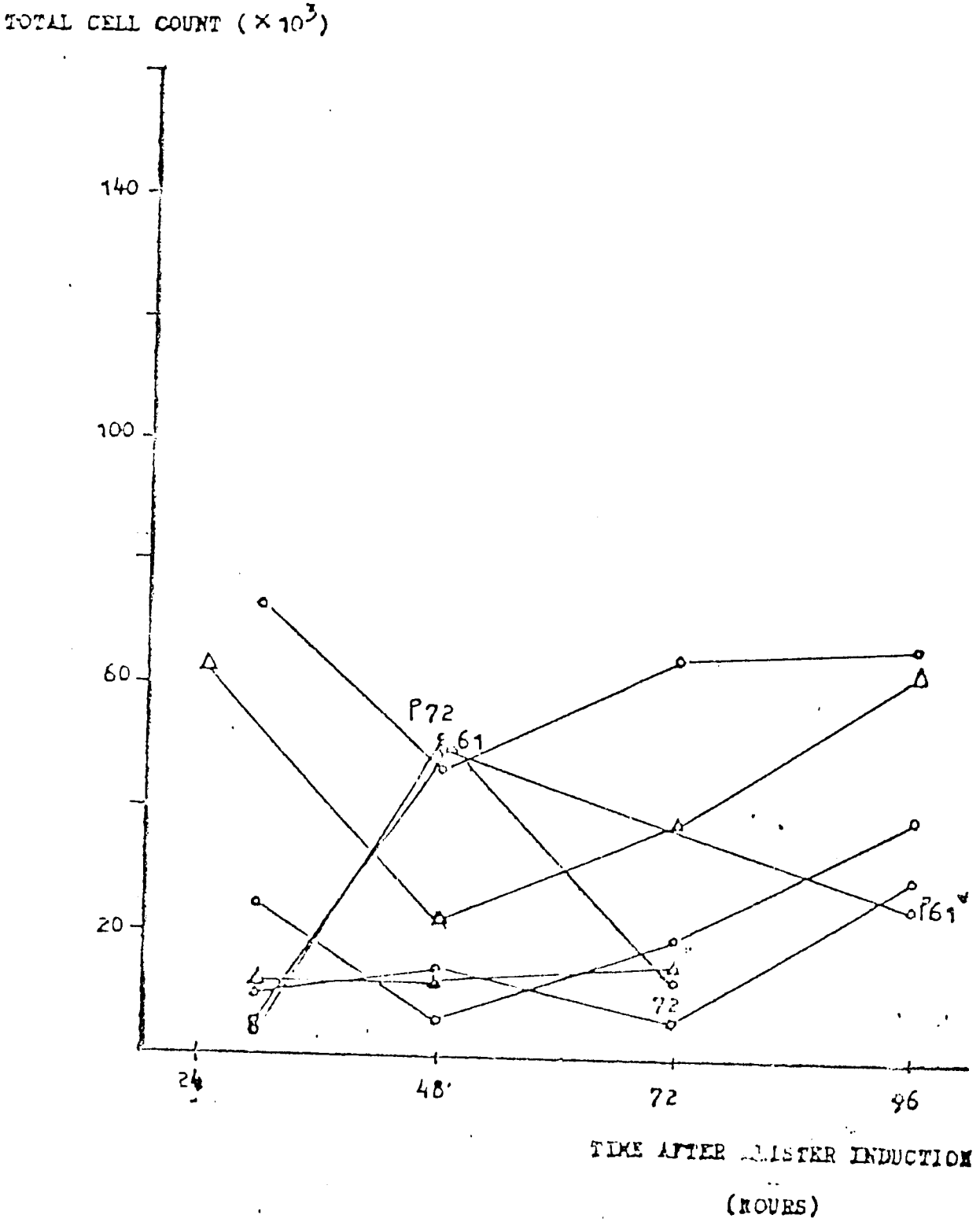


• Indicates patient number.

FIGURE 1C

TOTAL CELL COUNTS

ACTIVE BE(Δ) AND BT(O) E REACTIONS

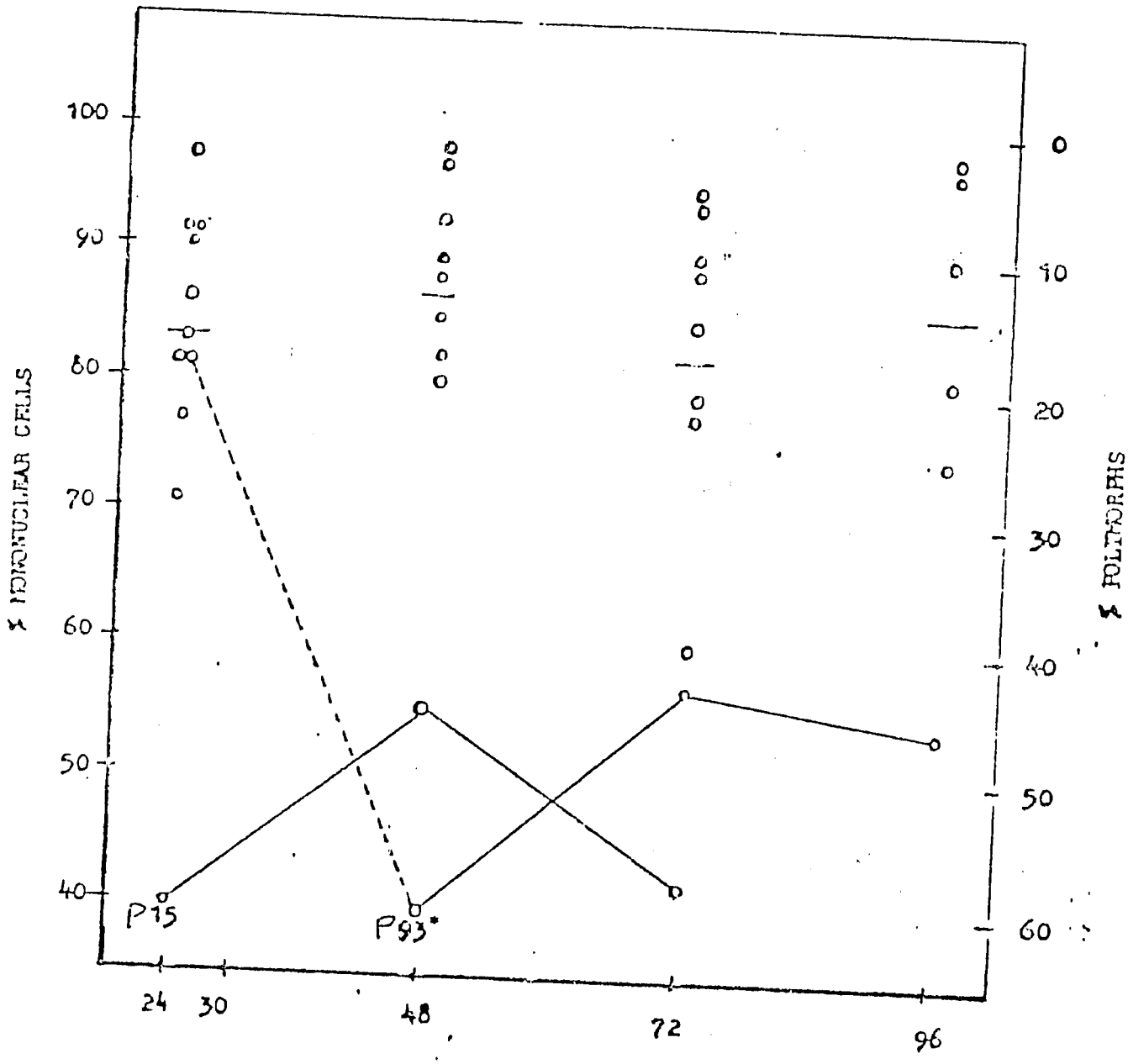


*Indicates patient number.

FIGURE 2A

MONONUCLEAR VS. POLYMORPHONUCLEAR CELLS

ACTIVE LL $\bar{\delta}$ REACTION



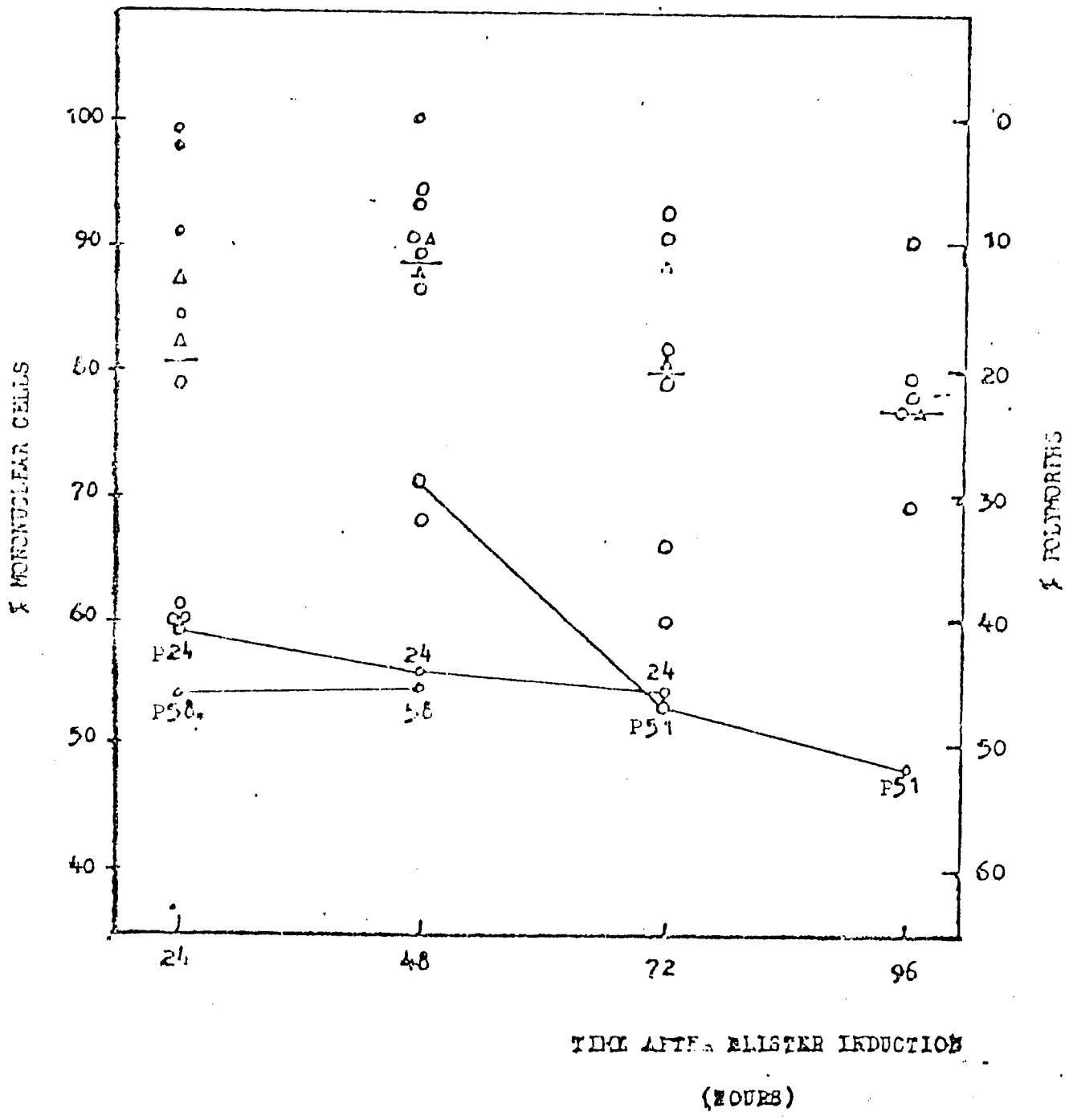
TIME AFTER BLISTER INDUCTION (HOURS)

*Indicates patient number.

FIGURE 2B

MONONUCLEAR CELLS VS POLYMORPHS

ACTIVE ML(O) & MN(Δ) E REACTIONS



*Indicates patient number.

FIGURE 2C

MONONUCLEAR CELLS VS POLYMORPHS

ACTIVE BT E REACTIONS

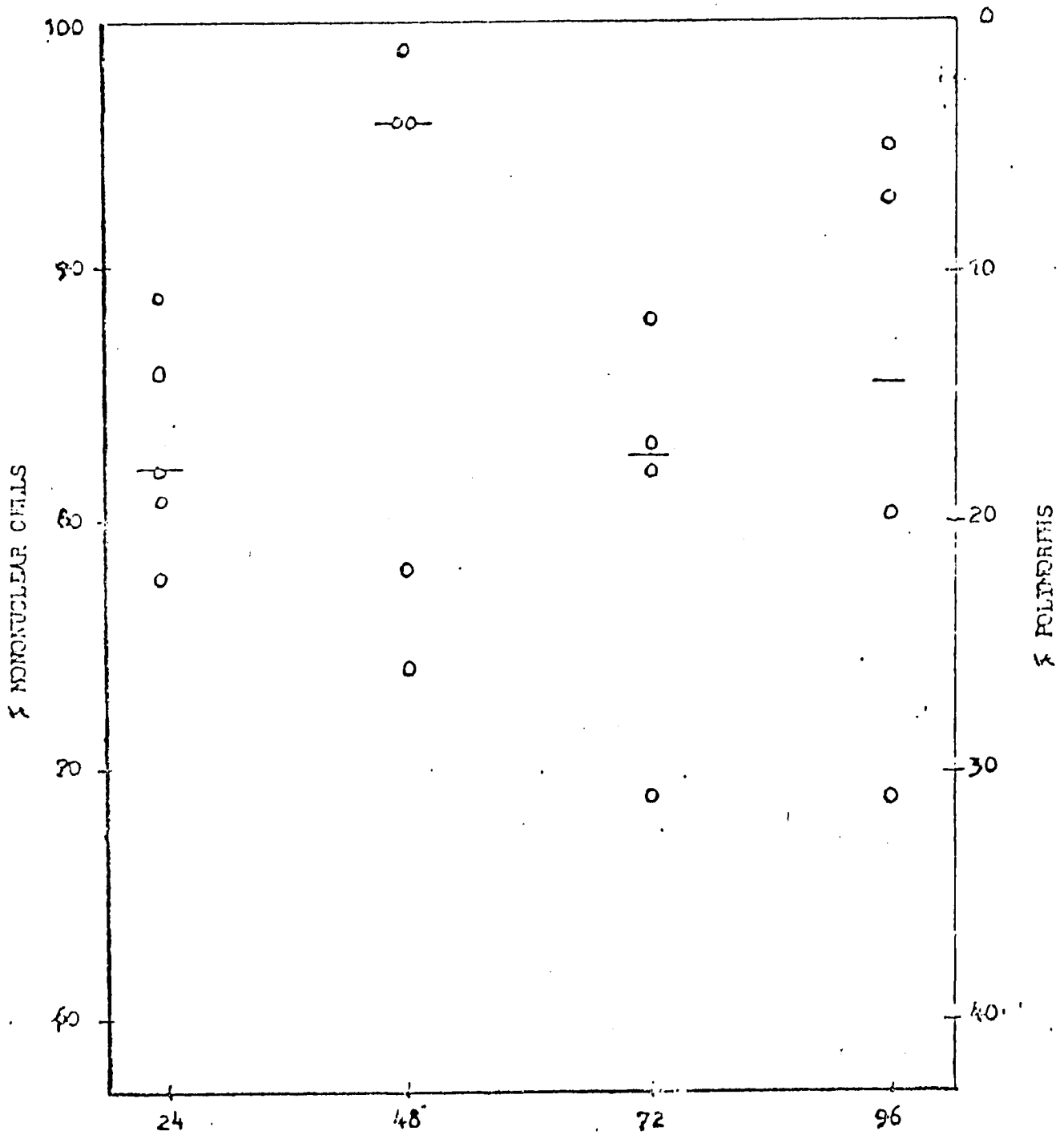
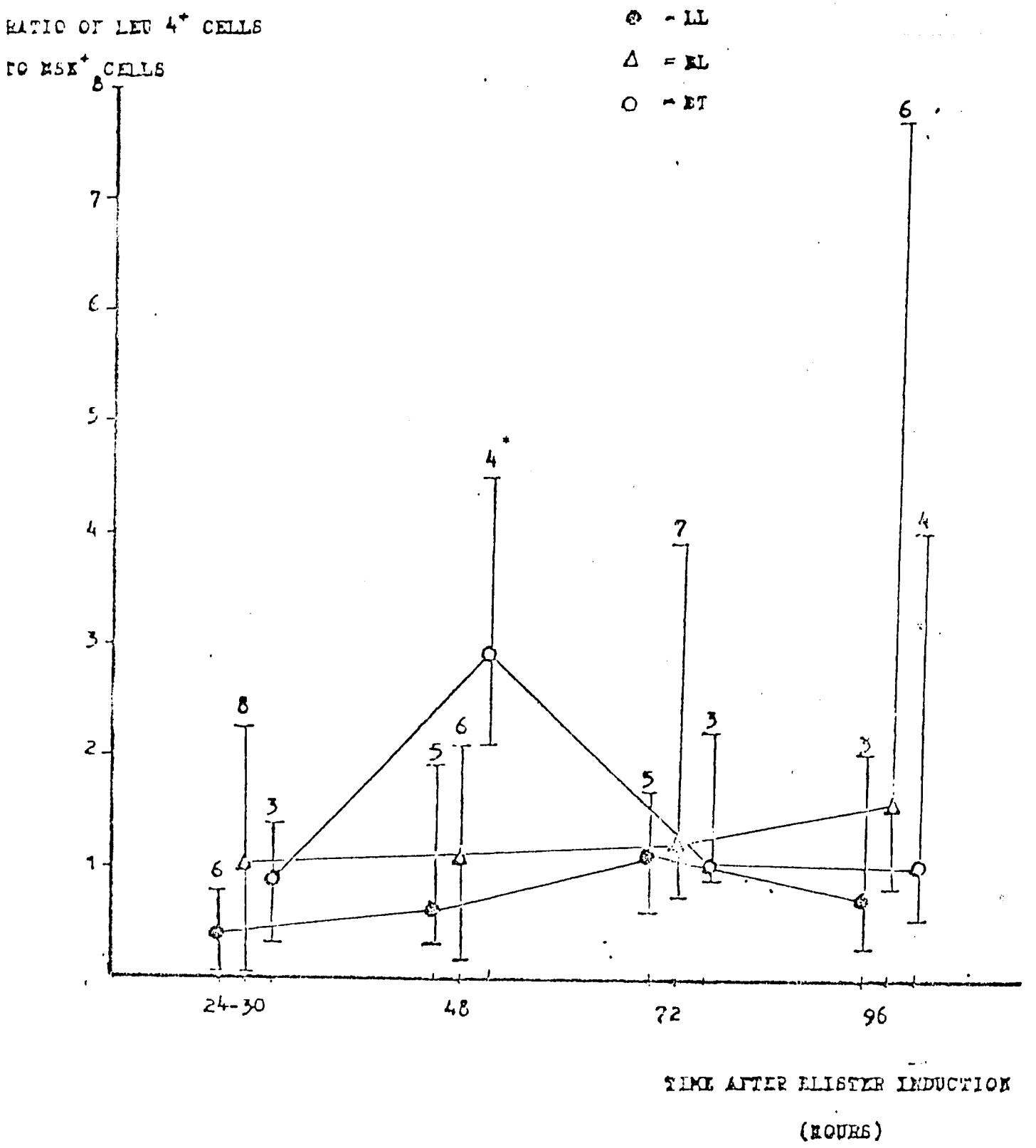


FIGURE 3

UNCOMPLICATED LEPROSY

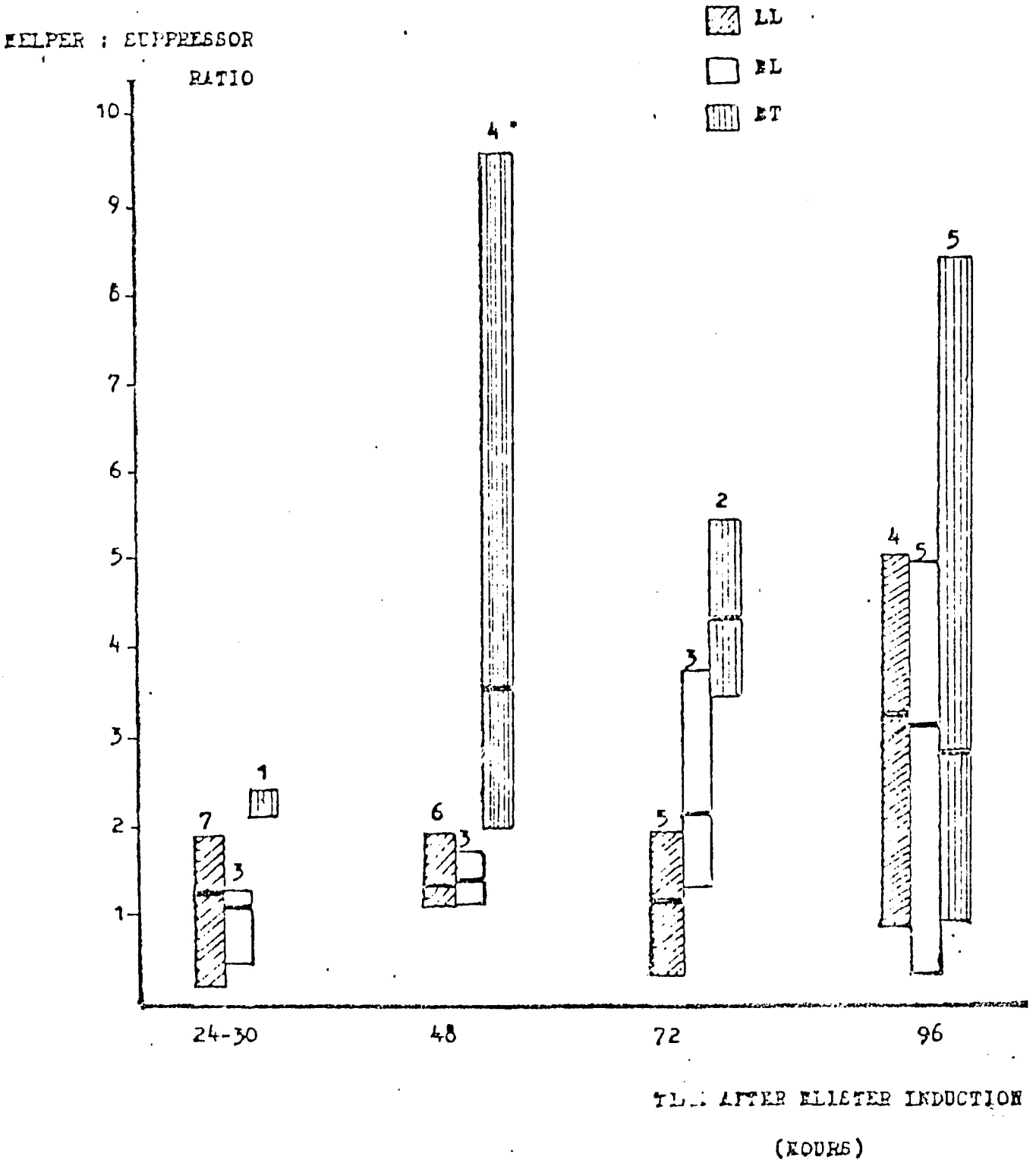
T : K RATIO IN BLISTERS



Indicates numbers of patients.

FIGURE 4

HELPER : SUPPRESSOR RATIOS : UNCOMPLICATED LEPROSY



Indicates numbers of patients.