International Journal of Leprosy

Volume 70, Number 4
Printed in the U.S.A.
(ISSN 0148-916X)

CORRESPONDENCE

This department is for the publication of informal communications that are of interest because they are informative and stimulating, and for the discussion of controversial matters. The mandate of this Journal *is to disseminate information relating to leprosy in particular and also other mycobacterial diseases. Dissident comment or interpretation on published research is of course valid, but personality attacks on individuals would seem unnecessary. Political comments, valid or not, also are unwelcome. They might result in interference with the distribution of the* Journal *and thus interfere with its prime purpose.*

AP-1 Is Present in Nuclear Extracts of Lymphocytes from Lepromatous Leprosy Patients

To the Editor:

Lepromatous leprosy (LL) patients present a limited T-cell *in vitro* proliferation to mitogens and to specific antigens, as well as a deficient interleukin-2 (IL-2) synthesis. The above mentioned is correlated with the presence of multiple *M. leprae* bacilli as an immunodeficiency state due, in part, to an inadequate activation of T cells ([1, 4, 5, 8]).

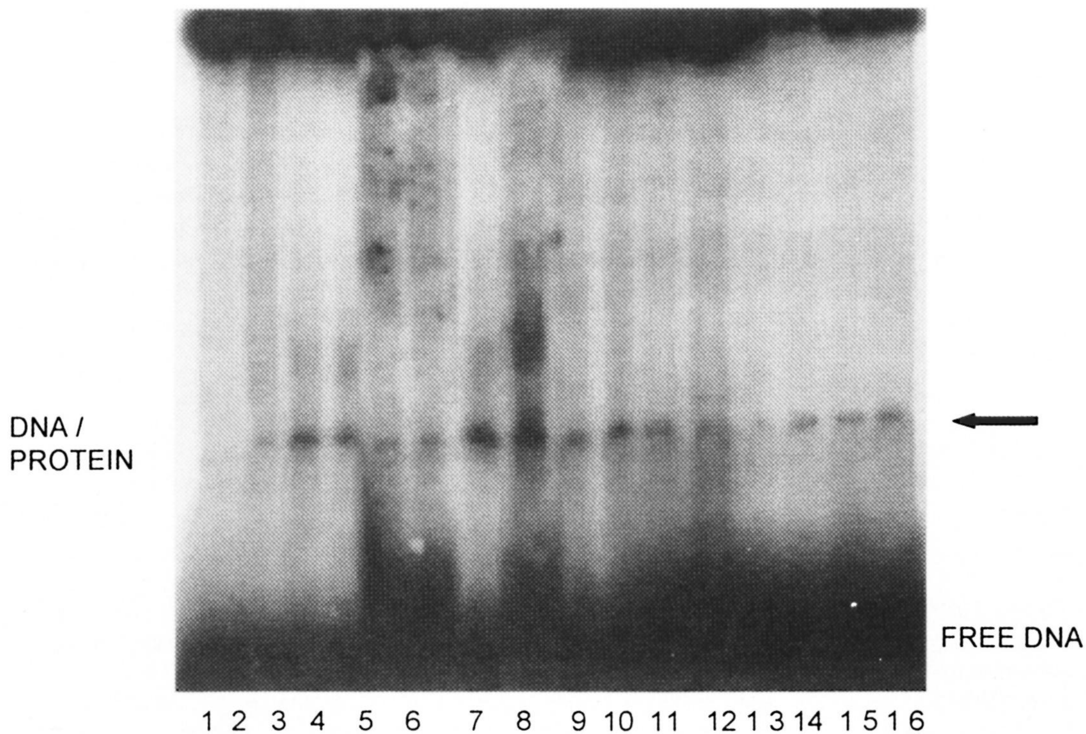
It has been shown in different models that T cells fail to proliferate due to a defect in IL-2 gene transcription ([7, 11]), through a deficient production of activator-protein-1 (AP-1), and other factors as well. AP-1 is an important and well studied, inducible transcription factor. AP-1 is a dimer protein containing the gene products of the *jun* (c-Jun, Jun-B and Jun-D) and *fos* (c-Fos, FosB, Fra-1 and Fra-2) gene families. Many signal transduction pathways involve the expression of c-fos and c-jun, formation of the dimer and union to the AP-1 consensus binding site, the oligonucleotide palindromic sequence. 5′-CGC TTG **ATG ACT CAG** CCG GAA-3′ ([6, 9, 10]).

In the present work, we looked for the presence of the AP-1 factor in nuclear extracts of PHA activated T lymphocytes from LL patients to acknowledge if the perturbed proliferation and IL-2 response in T cells correlated with a reduced DNA binding activity of this transcription factor.

Study subjects. Ten lepromatous leprosy (LL) patients (ages 20 years to 50 years), 3 females and 7 males from La Barca, Jalisco, México, were diagnosed as having LL according to international criteria ([12]). All were under irregular multidrug therapy treatment (dapsone, clofazimine, rifampin) according to World Health Organization (WHO) schemes, since diagnosis ([13]). All still presented positive bacilloscopy. The healthy control group consisted of unrelated subjects, from the same geographical rural area, sex-and-age (± 5 years) matched as the patient group.

Mononuclear cells. Heparinized blood (50 ml–100 ml) was obtained from each subject by venipuncture at the local hospital, transported to the laboratory in a 15°C ice chest and stored at 4°C overnight. The mononuclear cells were centrifuged at 400 × *g*, using Histopaque-1.077, washed and resuspended in RPMI 1640 culture medium (GIBCO, Grand Island, New York, U.S.A.) supplemented with 5% heat inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, penicillin 100 U/ml, and streptomycin 100 µg/ml. Finally, the cells were adjusted to 1×10^6 cells/ml ([1, 8]).

Proliferation and biosynthesis of IL-2 by T lymphocytes. Five µg/ml of phytohemagglutinin (PHA) was added to 2×10^5 mononuclear cells for culture. The cultures were incubated for 48 hr at 37°C in a mixture of 95% air and 5% CO_2. Thereafter,



THE FIGURE. Gel Electrophoresis Mobility Shift Assays (EMSA) showing complexes formed between AP-1 consensus site γ-P^{32} labeled oligonucleotides (AP-1c) and nuclear extracts (NE) of lymphocytes from LL patients and healthy controls (HC) subjects: Lane 1: Control without NE; lane 2: HeLa cell control NE; lanes 3–6: AP-1c + NE from cells of HC; lane 7–16; AP-1c + NE from cells of LL patients. Lanes 3, 5, 7, 9, 11, 13, 14 complexes of AP-1c + NE from cultures with medium. Lanes 4, 6, 8, 10, 12, 15, 16 complexes of AP-1c + NE from cultures stimulated with PHA.

each supernatant was assayed for IL-2 activity (ELISA) or pulsed with 1 μC of ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts, U.S.A., specific activity 6.7 Ci/μmol). After a 24-hr incubation period, the cells that had been pulsed were harvested, and the incorporation of ^{3}H-thymidine was measured in a Beckman beta counter. The results were expressed as counts per minute (cpm) and as stimulation index (SI) according to the following: SI = cpm of experimental (with PHA) ÷ cpm of medium (1).

IL-2 assay. For the quantitative determination of human IL-2 concentrations present in culture supernatant, a specific ELISA kit ("h-interleukin-2" ELISA kit from, Boehringer Mannheim, Germany) was used according to instruction from the manufacturer. Results were expressed as pg/ml of supernatant.

Preparation of nuclear extracts (NE). Under culture conditions, in 25 ml bottles, 5 $\times 10^6$ cells were incubated (Corning, New York, New York, U.S.A.), for 6 hours only, harvested by centrifugation and then the nuclear extracts were prepared according to the method described by Digman ([3]). The protein content of each extract was determined by the Bradford assay and the extracts stored in aliquots at –80°C until used in the Electrophoretic Mobility Shift Assay (EMSA). Hela cells nuclear extracts were prepared as a positive control for AP-1 presence.

Oligonucleotides. Two different Ds DNA oligonucleotides were labeled by T4 kinase phosphorylation reaction in the presence of γ-P^{32}-ATP (New England Nuclear, Boston, Massachusetts, U.S.A., specific activity 3000 μCi/mmol) according to the instructions from the manufacturer, and separated from unincorporated nucleotides by chromatography on G-50 Sephadex (Bio-Rad Laboratories, Hercules, California, U.S.A.). The oligonucleotide was 5′-CGC TTG **ATC ACT CAG** CCG GAA-3′. A

THE TABLE. *Counts per minute (cpm), IL-2 levels and Stimulation Index (SI) of cultivated activated T lymphocytes from LL patients and healthy subjects.*

Pair	LL patients			Healthy subjects		
	cpm/IL-2 (ng/ml)		SI	cpm/IL-2 (ng/ml)		SI
	Medium alone (unstimulated)	Medium + PHA (Stimulated)		Medium alone (Unstimulated)	Medium + PHA (Stimulated)	
1	805/98.7	3640/193.2	4.52	903/107	3150/440.8	5.22
2	1926/151.2	4809/142.2	2.49	1443/91.9	3366/161.5	2.33
3	1649/113.2	2028/64	1.23	202/123.6	756/1387	3.74
4	772/64.2	1825/131.2	2.36	645/78	1474/208.4	2.28
5	459/76.6	649/109.1	1.41	701/88.9	1483/82.9	2.11
6	4114/90.4	4978/425.66	1.21	3638/59.5	7121/131	1.96
7	4978/87.7	6091/118.8	1.22	3440/137	8684/127	2.52
8	8598/89.1	9078/116.3	1.05	4967/74	9642/109	1.94
9	239/167.7	526/80.1	2.20	623/116	2751/131.9	4.41
10	357/138.1	718/369.8	2.01	718/95	2619/287.3	3.65
Mean ± S.E.	2389 ± 860/ 107.7 ± 10.8	3434 ± 890[a]/ 180.07 ± 38.4[d]	1.97 ± 0.33[b]	1698 ± 529/ 97 ± 8	4104 ± 1006[c]/ 287 ± 47	3.02 ± 0.37

[a] No statistically significant difference compared to non-stimulated LL T cells.
[b] Statistically significant difference compared to healthy subjects p = 0.047.
[c] Statistically significant difference compared to non-stimulated healthy T-cells.
[d] No statistically significant difference IL-2 (PHA stimulated-T-cells) from LL compared with IL-2 (PHA stimulated-T-cells) from healthy subjects.
(Statistical analysis was carried out using the Student's *t* test.)

mutant oligonucleotide was used for heterologous competition assay, presented as "CA" → "TG" substitution in the AP-1 binding motif (Santa Cruz Biotechnology, Inc., Santa Cruz, California, U.S.A.). Non-specific competition was realized with growing amounts of poly (dldC) ([2]).

Binding reaction. The binding reaction (20 µl in HEPES, buffer pH 7.9) was performed with the same amount of nuclear protein in each reaction (10 µg) (equivalent to 500,000 cells) with 30 µCi (3 µl) of correspondent Ds ^{32}P-labelled oligonucleotide in the presence of 100 ng/µl of poly(dldC) as mentioned, to remove non-specific unions. The reactions were incubated on ice for 15 min.

Electrophoretic Mobility Shift Assay (EMSA). The above described samples were electrophorized on a 6% native Tris-borate-EDTA (TBE) polyacrylamide gel in 0.5 × TBE, at 200 volts, for 2 hr, at 4°C. The gels were dried under vacuum and exposed to autoradiography at –70°C using Kodak film (X-OMAT) during 24 hr ([2]).

Statistics. "BioStat Primer" statistical package was used to run "ANOVA" (Student "*t*" tests).

The Table presents experimental data of T-cell proliferation (expressed as cpm, as well as SI) and IL-2 levels. As expected, T cells from LL patients present a statistically significant ($p < 0.05$) lower stimulation index (SI = 1.97, ± 0.33) as compared to healthy controls (SI = 3.02, ± 0.37). Lymphocytes from LL patients have a tendency to produce lower levels of IL-2 as compared to healthy controls, (180.07 vs 287.47 ng/ml), nevertheless, no statistically significant differences were found.

The Figure shows the results of the EMSA. One can see the presence of AP-1 in the nuclear extracts of T lymphocytes from LL patients and healthy controls, stimulated with PHA or not. It is evidenced that all of them contain the AP-1 transcription factor as they provide the specific retardation band (indicated by an arrow).

The above mentioned leads us to conclude that the T cell unresponsiveness and low IL-2 levels observed in T cells of LL patients is not due to a defect in the production of the AP-1 transcription factor and, by inference, neither of Fos and Jun proteins.

We will study other transcription factors, such as NFkB, and/or NFAT, in order to know their role in activation of T lymphocytes and IL-2 synthesis from LL patients.

—Rosario Huizar-López, M.Sc.
Ann Santerre, Ph.D.

Departamento de Biología Culular y Molecular del Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Jalisco México. Apartado Postal 5-419. C.P. 45040 Colonia Chapalita, Zapopan, Jalisco, MEXICO.

—Vicente Madrid-Marina, Ph.D.
Oscar Peralta-Zaragoza, M.T.

Laboratorio de Inmunología del Instituto Nacional de Salud Publica (SSA) Cuernavaca, Morelos, México.

—Alma Villalobos-Arámbula, Ph.D.
Alfonso Islas-Rodríguez, Ph.D.

Departamento de Biología Culular y Molecular del Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Jalisco México. Apartado Postal 5-419. C.P. 45040 Colonia Chapalita, Zapopan, Jalisco, MEXICO.

Reprint requests to Alfonso Islas-Rodríguez. Apartado Postal 5-419. C.P. 45040 Colonia Chapalita, Zapopan, Jalisco, MEXICO.

Acknowledgment. M.Sc. Eduardo Gonzalez, Major of Jamay Jalisco. Dr. Genaro Paredes Romero, (SSA), La Barca, Jalisco, México. This study was supported in part by a grant from CONACYT #2578 P-M, and FOMES #98-15-12.

REFERENCES

1. Alfaro-Bustamante, F., Ramírez-Flores, G., Gonzalez-Mendoza, A., Islas-Rodriguez, A. E. and Fafutis-Morris, M. Effect of Phorbol myristate acetate (PMA) and ionophore A23187 on interleukin-2 levels and proliferation of activated T lymphocytes from patients with Lepromatous Leprosy. Int. J. Lepr. **65** (1997) 73–79.
2. Becker, H., Stengl, G., Steing, M. and Federlin, K. Analysis of proteins that interact with the IL-2 regulatory region in patients with rheumatic diseases. Clin. Exp. Immunol. **99** (1995) 325–330.
3. Digman, J. D., Levbovitz, R. M. and Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11** (1983) 1475–1489.
4. Fafutis, M. M., Mejia-Arreguin, S., Gonzalez-Mendoza, A. M., Morales-Ortiz, R. and Islas-Rodriguez Alfonso. Detection of IL-2 receptor by indirect immunofluorescence with anti-Tac monoclonal antibody on the surface of the T lymphocytes from patients with LL. Int. J. Lepr. **58** (1990) 126–128.
5. Fafutis-Morris, M., Alfaro-Bustamante, F., Gonzalez-Mendoza, A., Morales-Ortiz, R. and Islas-Rodriguez, A. E. Response to phytohemagglutinin of LL patients lymphocytes preincubated in culture media. Int. J. Lepr. **59** (1991) 480–482.
6. Folleta, V. C. Transcription factor AP-1, and the role of Fra-2. Immunol. Cell Biol. **74** (1996) 121–133.
7. Fraser, J. D., Irving, B. A., Crabtree, G. R. and Weiss, A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science **251** (1991) 313–316.
8. Islas-Rodriguez A., Morales-Ortiz, R., Fafutis-Morris, M., Gonzalez-Mendoza, A. and Ortiz-Ortiz, L. Deficiency in the biosynthesis of interleukin-2 (IL-2) and functional presence of the IL-2 receptor in lepromatous leprosy. Int. J. Lepr. **55** (1987) 566–569.
9. Jain, J., Loh, C. and Rao, A. Transcriptional regulation of the IL-2 gene. Curr. Opin. Immunol. **7** (1995) 333–342.
10. Jungu, J., Valge-Archer, V. E. and Rao, A. Analysis of the AP-1 sites in the IL-2 promoter. J. Immunol. **148** (1992) 1240–1250.
11. Kitagawa-Sakakida, S. and Schwartz, R. H. Multifactor *cis*-Dominant negative regulation of IL-2 gene expression in energized T cells. J. Immunol. **157** (1996) 2328–2339.
12. Ridley, D. S. and Jopling, W. H. Classification of leprosy according to immunity; a five group system. Int. J. Lepr. **34** (1966) 255–273.
13. Visschedijk, J., van de Broek, J., Eggens, H., Lever, P., van Beers, S. and Klatser, P. Review: *Mycobacterium leprae*—millennium resistant! Leprosy control on the threshold of a new era. Trop. Med. Int. Health **5** (2000) 388–399.