device to keep the testes cool and it may be cooler than the other areas of the body, which could explain the finding of leprosy lesions over the scrotum, while lesions over the shaft and prepuce are very rare. Underreporting of these cases is either due to a hesitancy of the patients to expose, or due to the reluctance of physicians to examine the genitalia ([8]). It is also possible that the temperature dependency of *Mycobacterium leprae* is not absolute, as evident by the facts that *M. leprae* do survive in warm sites such as bone marrow ([11]), lymph nodes ([6]), the liver ([5]) and palms and soles of the feet ([1]).

In the modern era of HIV/AIDS, when patients, as well as physicians, are very concerned about lesions in the genital area, especially in high-risk patients, a wider range of suspicion should be kept in mind, including Hansen's disease, while examining these patients. In our case, the lesions were present over the scrotum, in addition to a lesion over the prepuce. The nodular lesion over the prepuce was quite large causing phimosis and was, consequently, confused with a sexually transmitted disease.

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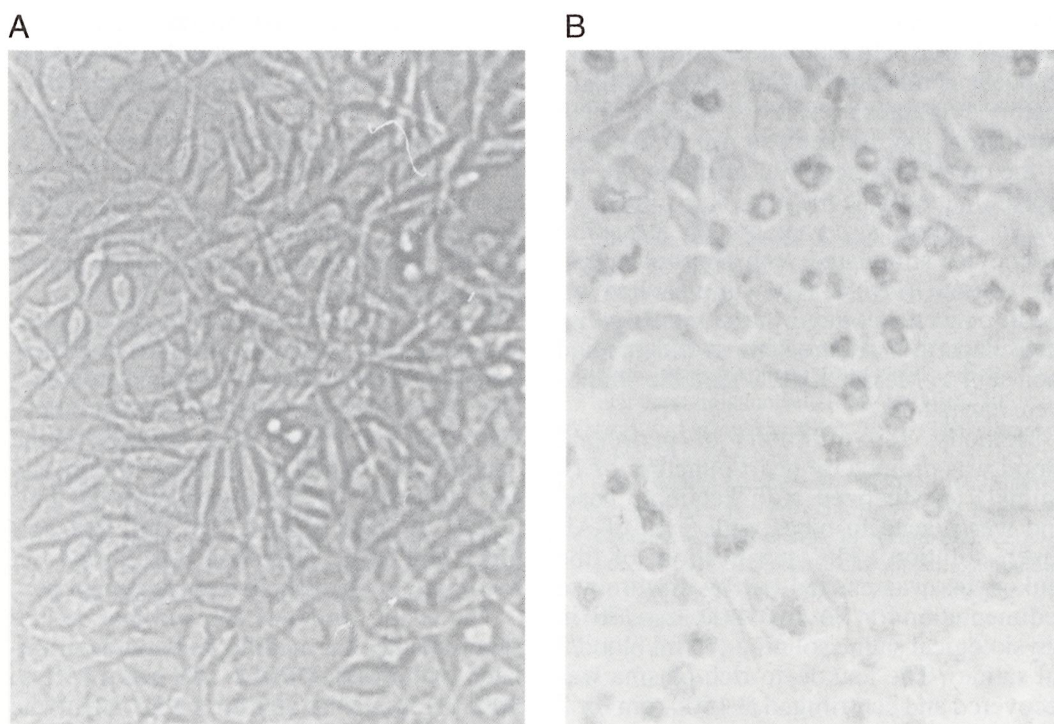
Detection of a Tumor Necrosis Factor-Like Activity in Culture Supernatants of Armadillo Leukocytes

TO THE EDITOR:

Tumor necrosis factor (TNF) is one of the main cytokines involved in the immune response. It was discovered by Old in 1985 ([10]) as a factor with cytotoxic activity on tumor cells that is liberated in mouse serum in response to lipopolysaccharide (LPS) injection. TNF is released by several cells of the immune system, such as activated T lymphocytes and mastocytes, NK cells, monocytes, macrophages and neutrophils ([3]). Identification and characterization of this molecule is by means of enzymatic im-



THE FIGURE. Cytotoxic effect of the culture supernatants of armadillo leukocytes stimulated with PMA on the L-929 cells. **A** = culture supernatants of armadillo leukocytes not stimulated with PMA. **B** = Culture supernatants of armadillo leukocytes stimulated with PMA (50 ng/100 ml) for 24 hr.

munoassay and/or bioassay evaluating the cytotoxic effect on cell lines, such as L929, WeHI164, L-M, OVCAR-3 and Pk15 ([4,5,7]). These assays have allowed the demonstration of the presence of TNF in various species of vertebrate and invertebrate animals ([1,2,9,11]). The biochemical characterization of this cytokine among the diverse animal species has revealed similarities in both structural features and biological properties whereby it has been considered as a molecule conserved evolutionarily ([1,2,9,11]).

The armadillo (*Dasypus novemcinctus*) has been considered as a feasible animal model to study the infection with *Mycobacterium leprae* ([8]), however, this has not been completely possible due to little knowledge of their immune response mechanisms. Because *M. leprae* is an intracellular microorganism, the host cellular immune response

THE TABLE. *Effect of PMA on TNF production by armadillos leukocytes and U-937 cells.*

Stimulation with:	U-937 cells[c]		Armadillo leukocytes[c]	
	TNF (pg/ml)[a]	Cytotoxicity (%)[b]	TNF (pg/ml)[a]	Cytotoxicity (%)[b]
Medium alone	0.0	0.0	0.0	2.68 ± 0.5
LPS (1 μg/ml)	0.0	0.0	0.0	5.11 ± 1.4
PMA				
25 ng/ml	28 ± 7.5	47.8 ± 10.1	0.0	14.9 ± 1.0
50 ng/ml	10 ± 1.4	55.8 ± 14	0.0	27.7 ± 3.5
LPS + PMA (25 ng/ml)	191 ± 6.9	48.8 ± 11.7	0.0	28.9 ± 6.0

[a] TNF-α from cell culture supernatants of 24 hr was measured by ELISA method, sensitivity of 1 pg/ml.
[b] Cytotoxic activity from cell culture supernatants of 24 hr was evaluated in L-929 cells bioassay.
[c] Mean ± standard deviation of 10 separate assays.

plays an essential role for the elimination of this pathogen. The activation of this cellular response is mediated mainly by pro-inflammatory cytokines as TNF ([2]). Because TNF production has not been shown in the cells of the immune system of armadillos, we decided to investigate the presence of this factor in culture supernatants of armadillo leukocytes stimulated with phorbol myristate acetate (PMA). The nine-banded armadillos were captured in the municipality of Chilapa in Guerrero state in Mexico and then sent to Mexico City, where the studies were carried out.

Methods: Ten milliliters of peripheral blood was drained by heart puncture of 10 animals anesthetized with ketamine base, and was placed in tubes with 5 ml of Alsever solution. The purification of the leukocytes was carried out by erythrocyte sedimentation for 1 hr in 6% dextran '70' in physiological saline solution (10 ml blood/3 ml saline). The leukocyte-rich plasma was recovered and centrifuged at 1500 rpm for 7 min. The resulting cell pellet was washed twice in Alsever solution. The final leukocyte pellet was resuspended at 2×10^6 cells/ml in RPMI-1640 medium (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) supplemented with 10% fetal calf serum and antibiotics (RPMI-complete). One million cells was added to each well of a 24-well flat-bottom microplate. Then, the armadillo leukocytes were stimulated with phorbol myristate acetate (PMA) and/or LPS (Sigma). The microplate was incubated at 37°C with 5% CO_2. The culture supernatants were recovered after 24 hr and maintained at –70°C until use.

The detection of TNF was carried out by a cytotoxicity bioassay using L-929 cells as previously described ([6]). Briefly, 50 µl of the culture supernatants was added in each well in triplicate in 96-well flat-bottom microplates that contained 20×10^3 L-929 cells/well. Then, 50 µl of RPMI-complete with 1 µg/ml of actinomycin D (Sigma) was added to each well. Culture supernatants of U-937 cells stimulated under the same conditions as those of the armadillo leukocytes were used as the control. The microplate was incubated at 37°C for 24 hr in 5% CO_2. Supernatants were aspirated and 100 µl of methanol was added. After 7 min of incubation, 50 µl of 2% crystal violet was added. After 10 min, the microplates were washed five times with 200 µl per well with phosphate buffer solution (PBS). Then 100 µl of 33% glacial acetic acid was added. Finally, the microplates were read in a microplate reader at 600 nm for absorbance (Ceres 900Hdi, Bio-Tek Instruments, Inc., Winooski, Vermont, U.S.A.). Results were reported as percentage of cellular cytotoxicity.

In order to confirm the results of the bioassay, an ELISA commercial kit for the determination of human TNF-α (Roche Molecular Biochemical, Mannheim, Germany) was used. The ELISA assay is based on the quantitative "sandwich enzyme immunoassay" principle, two mouse monoclonal antibodies directed against two different epitopes of TNF-α. Detection was carried out according to the manufacturer instructions.

The results obtained showed that culture supernatants of armadillo leukocytes stimulated with PMA or PMA-LPS had a 14% to 30% cytotoxic activity on L-929 cells (The Table and The Figure). A similar effect was observed in culture supernatants of U-937 cells stimulated with PMA or PMA-LPS, but with a cytotoxic activity of 37% to 70% on L-929. The quantification of TNF by means of the ELISA method showed the presence of TNF in the culture supernatants of U-937 cells, while in the culture supernatants of armadillo leukocytes this cytokine was not detected. However, it is necessary to mention that the ELISA test was carried out using monoclonal antibodies that only recognize specific epitopes for the human TNF-α and does not react with TNF-α from a mouse, rat, dog, pig, rhesus monkey or baboon. For this reason it would be necessary to purify and to characterize this molecule to demonstrate that the cytotoxic activity observed was due to a factor similar to human TNF-α.

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