4 STUDY OF THE EXPRESSION AND ROLE OF INFLAMMATORY CYTOKINES IN IMMUNOPATHOLOGY OF LEPROSY

4.1 MATERIALS AND METHODS

4.1.1 Localisation of TNFα in the skin biopsies by Immunohistochemical studies

Reagents used:

MoAb 52B83: mouse monoclonal against human TNFα
(A gift from Dr. Burmann, Netherlands)

ABC/APAAP kit Vector Laboratories, USA
AEC Sigma
DAB Fluka laboratories
Fuchsin Red Biogenex laboratories
OCT compound Miles Scientific

Specimens: Skin biopsies were collected either by 4 mm biopsy punch (Baker Cummins, Florida, USA) or by open biopsy from the border of the active representative lesion site of the patients. The biopsy tissue was immediately placed in aluminium foil and snap-frozen by immersing in an isopentane/liquid nitrogen slush for 1-2 minutes. All the biopsy tissues were stored at -70°C till further processing.

Cryosectioning: OCT compound (Tissue Tek, Miles Scientific) was put on the cork support and the tissue section was placed in proper orientation over the jelly. The cork support along with the tissue section was dipped in liquid nitrogen or kept inside the chamber of cryostat (-25°C) to allow the OCT compound to solidify. More OCT compound was poured over the tissue to embed the block completely and was fixed over the metal specimen holder with OCT. The block was then transferred to the cryostat chamber and allowed to equilibrate to the temperature of the cryostat for at least one-half hour before sectioning began. The specimen holder was mounted in the Reichert Frigocut cryostat
and 5μm thick sections were cut manually at -25°C. The section was transferred on to the polylysine-coated slide by gently lowering it over the knife surface. The section melted onto the slide when came in contact. The slide was then air-dried at room temperature for 1-2 hours and then stored at -20°C.

**Immunostaining:** Before starting the immunostaining, the sections were fixed in acetone at room -20°C for 10 minutes. The slides were washed in 10 mM PBS with powdered milk (2gm/l) for 20 minutes with continuous stirring with a tiny magnetic bar over magnetic stirrer. Pre-incubation with normal blocking serum (Normal Horse serum in 1:50 dilutions) was done subsequently for 20-30 minutes in humid conditions at RT to block non-specific sites. Excess normal blocking serum was flipped and the surrounding area of sections were dried with tissue paper except the control slide where no first antibody was added.

For TNF-α staining mouse monoclonal antibody 52B83 (5 μg/ml) against human TNF-α was used as first specific antibody in 1:500 dilution in 10mM PBS-0.3% BSA. 100 ml of the antibody solution was put over the sections and incubated in humidified chamber at RT for 1 hr. The slides were then washed in PBS-milk solution with stirring for 10 minutes. Biotinylated anti-mouse IgG (H+L) affinity purified antibody made in horse from Vector laboratories from Vectastain Elite ABC kit was used in 1:200 dilution in 1:70 Normal horse serum in 10mM PBS. Slides were incubated with this biotinylated antibody for 30 minutes in humidified chamber at RT followed by washing for 10 minutes. The endogenous peroxidase content of the skin sections was quenched by dipping the slides in 0.3% H₂O₂ for 30 minutes. The sections were then washed for at least 20 minutes before addition of Avidin-Biotin peroxidase conjugate (Vectastain elite ABC kit from Vector laboratories) ; Reagent A and Reagent B in 1:1 ratio in 1:50 dilution in 10 mM PBS buffer for 30 minutes. Washing was done with PBS-milk for 10 minutes before peroxidase revelation step by substrate.

In initial experiments, DAB (3,3 diaminobenzidine tetrachloride) was used for revelation. DAB solution was made by dissolving 6 mg of DAB in 10 ml of 50 mM tris-Hcl buffer to which 10 μl of 30% hydrogen peroxide was added. 4-6 drops of DAB
solution was placed onto each specimen and incubated for 3 minutes. The slides were washed in distilled water for 5 minutes and then counterstained with Hemalun stain for 8 minutes followed by washing in tepid water for 3 to 5 minutes. The sections were dehydrated in graded alcohol (50%, 60%, 70%, 80%, 90% and 100% serially) and then cleared in xylol. The slides were mounted in DPX. DAB gave a brown end product.

AEC (3-Amino-9-Ethyl carbazole from Sigma) was used in subsequent experiments which gave rose-red alcohol soluble end product. 4 mg of AEC (Sigma) was dissolved in 1 ml of N,N dimethyl formamide. While stirring, 14 ml of 100 mM acetate buffer pH 5.2 was added followed by 15 ml of 30% hydrogen peroxide. The mixture was filtered. 4-6 drops of AEC solution was placed onto each specimen and incubated for 20 minutes at room temperature. The slides were washed in tap water for 5 minutes and then counterstained with Meyer’s Haematoxylin stain for 8-10 minutes. The slides were air-dried and then mounted in DPX or Eukit.

Fuschin Red - was also tried which is insoluble in alcohol and other organic solvents, allowing for the specimens to be dehydrated before coverslipping. The intensity was greater than other compounds. This substrate was used with APAAP (Vector) kit instead of ABC system. Levamisole was added with substrate to block endogenous alkaline phosphatase. The dilution of second antibody was done in PBS buffer containing 10% normal human serum to reduce non-specific binding.

4.1.2 Expression of TNFα in the skin biopsies by in situ hybridization studies

Materials:
- cDNA: Gift from Dr. Kossodo, Geneva
- BSA: Sigma
- DTT: Sigma (dithiothreitol in 1X PBS)
- 3NTP-U: Sigma
- 35S UTP: Amersham
- RNAsin: Boehringer
- T 7 RNA Polymerase
DNAse Boehringer

**Transcription Buffer (TB 10X)** (for 100 ml)

- 200 mM NaCl 5M 4 ml
- 400 mM Tris-Hcl 1M 40 ml  (pH-7.5)
- 60 mM MgCl$_2$ 1M 6 ml
- 20 mM Spermidine 1M 2 ml
- Autoclaved double distilled water 48 ml

**STE = TEN 9** (for 200 ml)

- 100 mM NaCl 5M 4 ml
- 20 mM Tris-HCl 20M 4 ml  
  pH 8.8
- 1 mM EDTA 0.2M 1 ml  
  pH 7.4
- Autoclaved DDW 191 ml

**Tissue preparation**: Biopsy specimens were collected and frozen as described in previous section.

**Slide preparation**: Glass slides for use of *in situ* hybridization were first put in Hcl for 30 min at RT followed by rinsing with sterile distilled water twice. Then the slides were put in Ethanol 95 % at RT for 30 minutes and air-dried. Afterwards the slides were put in poly L lysine (100 mg/ml in sterile distilled water) at RT for 30-60 min (the poly L lysine can be used several times and must be stocked at 4°C). The slides were rinsed twice with sterile distilled water and sterilised in hot air oven - 120°C for 1 hr.

**Fixation**: The sections of 5 m were put on the processed slides and slides were with glutaraldehyde (4% PBS) for 5 min.
Storage: The slides were stored in 70% ethanol at 4°C till in situ hybridization experiment.

Radiolabelling of probe (Transcription)
Following chemicals were incubated at 37°C water bath for 30 min before the procedure.

10x TB
BSA(2 mg/ml)
DTT(30 mg/ml)=0,2 M
3NTP-U 5mM
35S UTP

RNAsin
T7 RNA polymérase
DNase

-Mixing was done in an eppendorf tube in the following order

10x TB 1ml
DTT 0.2 M 0.5 ml
RNA.sin 0.5 ml
3NTP-u 1 ml
BSA (2 mg /ml) 0,5 ml
S35 UTP(50 uCi) 5 UL
DNA linearised 1 ml
T7 RNA Polymerase 5U = 0,5 ml

The tube containing the mixture was put immediately in 37°C waterbath for 40 minutes. Then to it 0.5 ml SP6 polymerase (5m/ml of DNA) was added followed by 40
Localisation of TNF-α in the skin

72 min incubation at 40°C. To this 1 ml DNase was added followed by 20 min incubation at 37°C. Then 40 ml Rstop was added. Total DNA was extracted with phenol chloroform (50 ml of CHCl₃ 2X. Centrifugation for 3 min) after wash with 50 ml of TEN9. The G50 column was prepared by spinning the spin column at 1.2 K for 5 minutes and the probe (100 ml final after extraction) material was put in the column very slowly. The column was centrifuged at same speed (1.2 K) for 5 minutes. The column was washed with 100 ml of TEN9. Total of 200 ml was obtained on the final to which 1 ml YtRNA and 20 ml (1/10 of the final volume) of NaOAc 3M and 600 ml (3 vol) of 100% Ethanol. The probe was kept at -80°C for overnight. Then the tube was centrifuged for 15 min at 4°C and the supernatant was aspirated out. The pellet was resuspended in 100 ml hydrolysed buffer for 2 hrs at 60°C to which 10 ml of salt (NaOAc 3M) and 300 ml (3 vol.) of 100% alcohol was added and the tube was kept at -20°C for overnight for precipitation. Then, the tube was centrifuged for 15 minutes at 4°C, supernatant aspirated and 300 ml Ethanol 70% was added. The tube was centrifuged for 15 min at 4°C and the pellet resuspended in 200 μl of distilled water. The count was determined by counting 1 μl of the radiolabelled probe in scintillation fluid in the beta-counter.

Protocol for in situ hybridization

The slides were taken out of the alcohol and washed with 2xSCC for 2 min at RT twice. Then the slides were treated with Triethanolamine 0.1 M (250 ml) + 625 ml anhydride acetic acid for 10 min at RT. The slides were washed with 2xSCC for 1 min at RT followed by washing with PBS for 1 min at RT. Then the slides were treated with Tris-glycine 0.1 M 30 min at RT(250 ml) followed by washing with 2xSCC for 10 min at RT. Following this the washing was done with 2XSSC 50% formamide at 56°C for 20 minutes. Then the probe was put on the sections and the tissue area was covered with the coverslip and incubated for 3h at 48°C in humidified chamber (normally 20 μl of the probe was used per slide with a count of 2 X10⁶ cpm). After incubation the coverslips were taken off from the slide and were rinsed with in 2xSSC-50% formamide overnight followed by washing with 0.2xSSC-50% formamide 56° for 30 min. Then the slides were rinsed 4 times in 2xSSC at RT and then 200 μl/slide (20 μg/ml RNase A) RNase
was put for 30 min at 37°. The slides were again rinsed in 2xSSC-50 % formamide 5 min 56° following four rinses with in 2xSSC at RT. Then slides were dehydrated in graded alcohol of 60 %, 80%, 94% and allowed to dry.

**Detection of hybridization probe:**

The Kodak photo emulsion was diluted 1/1 by weight in water and put in water bath at 45° in dark room for 1 hr to melt. The *in situ* slides were dipped in photo emulsion to have an uniform layer of coating and the slides were left in a dark box for a period of 8 hours to weeks until development.

Kodac pl12 in concentration of 10% for 2 min 30 sec was used as revealtion solution in dark room for the slides and then washed in distilled water. The slides were fixed by 30% Na thiosulphate solution (30 gm/dl) for 5 min and washed in tepid water for at least 10 minutes to get rid of emulsion coating.

26. Giemmsa stain (5%) was used for 10 min for staining the nuclei and then the slides were scanned in light microscopy for silver grains concentrations.

**4.2 RESULTS**

**4.2.1 Localisation of TNFα in skin biopsies**

The immunohistochemical studies for TNFα showed positive staining in the macrophages and infiltrating mononuclear cells. Fig. 4.1.1 to Fig. 4.1.6 are representative microphotographs of the many experiments tried. Fig. 4.1.1 represents a LL case showing a few infiltrating cells in epidermis positively stained. In each set the microphotograph of the sister section used as control without treatment of primary antibody. In each case always one sister section was treated with all the reagents excluding the primary antibody. In Fig. 4.1.1 the epidermal cells shows brown colour due to melanin pigments which are rather granular whereas DAB positivity is homogenous. Fig. 4.1.2 demonstrates presence of abundant *M. leprae* in the same biopsy stained with Fite-Faraco' staining. Fig. 4.1.3 represents another LL patient showing positive cytoplasmic reaction for TNFα in giant cells and other infiltrating cells of monocytic lineage. Fig. 4.1.4 is of another LL patient showing mild to moderate
Fig. 4.1.1 Microphotograph of skin from LL case (KUD-8) showing a few infiltrating mononuclear cells in epidermis cells positively stained with anti-TNF-α antibodies (top). The control section with negative reaction without the treatment of primary antibody (bottom). [ABC, 500x]
Fig. 4.1.2 Microphotograph of skin from LL case (KUD-8) showing bacilli in clusters or lying scattered in the section. [Fite-Faraco's stain, 1000x]
Fig. 4.1.3 Section of skin from LL case (CHD-27) stained for TNFα. Positive cytoplasmic reaction in dermis (top). Control section stained in identical manner except the primary antibody has been replaced by NHS, there is no reaction (bottom). [APAAP, 400x]
Fig. 4.1.4 Photomicrograph of skin from LL patient (SHY-36), showing mild to moderate immunostaining for TNFα in the infiltrating cells in dermis (top). The corresponding control showing absence of reaction when primary antibody was replaced by NHS (bottom). [APAAP, 250x]
Fig. 4.1.5 Microphotograph of skin from LL patient (SHY-36) showing moderate to marked immunostaining for CD 68 (surface marker for monocyte) in an infiltrating area in dermis. [APAAP, 500x]
Fig 4.1.6 Section of a skin from TT patient (KKS-1) showing positive reaction for TNFα in a small-size blood vessel (top). The corresponding control without treatment of primary antibody shows no reaction (bottom). [APAAP, 250x]
Fig. 4.2.1 Photomicrograph of *in situ* hybridization of skin from LL patient (KUD-8) showing infiltrating cells with silver grains representing mRNA for TNFα counterstained with H & E. [ISH, 640x] exposure - 8 hrs.
Fig. 4.2.2 *In situ* hybridization of section of skin from TT (RAS-9) showing intracytoplasmic silver grains in a small granuloma counterstained with H & E. [ISH, 640x] exposure time - 8 hrs.
immunostaining for TNFα in the infiltrating cells in dermis. Fig. 4.1.5 is of same LL patient as of Fig. 4.1.4 where marked positive membranous reaction to CD 68 (marker for macrophages) is shown. Fig. 4.1.6 shows a TT patient's skin showing positive reaction for TNFα in a small-size blood vessel.

4.2.2 Expression of TNFα mRNA in skin biopsies

Preliminary results showed more concentrations of silver grains in the infiltrating cells of TT and also LL categories. Fig. 4.2.1 and Fig. 4.2.2 represents a LL and TT patient's skin section showing increased accumulation of silver grains in the infiltrating cells.

4.3 DISCUSSION
In immunohistochemical study of skin sections of LL, infiltrating monocytes were positive for TNFα indicating these cells to be the major contributors to elevated serum TNF in LL and reactional cases. However, this part of the study will have to be enlarged before a meaningful conclusion can be drawn. Other workers have similarly reported increased synthesis or accumulation of TNFα in the granulomatous infiltrates. Preliminary results of in situ hybridization using riboprobe for TNFα are indicative of increased expression of TNFα mRNA positive cells in the mononuclear cells of the granulomatous infiltrate and glial cells of the peripheral nerves (Sullivan et al. 1991; Yamamura et al. 1992).