SUBJECTS AND METHODS
**Subjects:**

One hundred and twenty-five TA patients and ninety age and sex matched healthy individuals as control subjects were selected for this study, which was approved by the Ethics Committee of Sanjay Gandhi Post-Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India. The diagnosis of TA was established on the basis of clinical, laboratory and angiographic findings of the disease. All patients fulfilled at least 3 of the American College of Rheumatology Criteria 1990 for TA (Arend et al. 1990). These include (i) bruits over subclavian arteries or aorta, (ii) decrease or absent brachial artery pulse and (iii) systolic blood pressure difference of >10 mm Hg between the arms. Finally in each case, the diagnosis of TA was confirmed by angiography and all the patients were found to have angiographically proven disease.

Among the patient group 58 were female and 39 were male and mean age of the patients at the time of diagnosis was 27±11 years (range: 12-45 years). Most of the control subjects were patient relatives and friends residing in the same locality as of the patient while some were volunteer donor or paramedical staff of SGPGIMS, Lucknow, India.

Disease activity of the patients was determined by the following criteria. (i) systemic features like fever, arthralgias, myalgias or weight loss of unknown cause; (ii) carotidynia (painful arteries); (iii) elevated ESR (> 30 mm/hr) and (iv) CRP (> 0.6 mg/dl) level. A patient was considered to be in the active stage if 2 or more of these criteria were present along with other features of the disease while remission was defined absence of inflammatory features, normalization of ESR and CRP and no appearance of new or progression of pre-existing lesions (Nityanand et al. 1997b).
Isolation of PBMCs and Plasma:

Peripheral blood mononuclear cells (PBMCs) were isolated from a total of 58 TA patients and 40 healthy controls at different time periods during the visits of the patients to hospital and days of different cellular experiments. The cells of 10 patients & 10 controls, 22 patients & 12 controls and 26 patients & 18 controls were used to study cytokine gene repertoire of PBMCs, T-cell cytokine profile and T-cell proliferation to HSPs, respectively.

Isolation of PBMCs was carried out by standard ficoll-hypaque (Histopaque: 1.077; Sigma, St Louis MO, USA) density gradient centrifugation method standard as per manufacture’s instructions. Briefly, after informed consent 5-10 ml of venous blood was taken from each subject in a sterile glass tube containing 50 IU of preservative free heparin (Sigma) and immediately stored at 4°C. Within one our of sample collection the blood was diluted with an equal volume of phosphate buffered saline (PBS, pH 7.4), loaded over appropriate volume of ficoll-hypaque and centrifuged at 400g for 30 minutes. The isolated mononuclear cells at the interface of the blood and ficoll was harvested into a fresh tube containing PBS and washed twice at 200g for 10 minutes. The purified cells were finally counted and either suspended at a concentration of 1-2.5 x 10^6 cells/ml in complete medium consisting of RPMI-1640 supplemented with 3 mM of L-glutamine, 10mM of N-2-Hydroxyethyl Piperzine-N-2-ethanesulphonic acid (HEPES) buffer, 1 mM of sodium pyruvate and 10% of heat inactivated fetal bovine serum (FBS) and bacteriostatic levels of penicillin-streptomycin (all from Gibco BRL, Grand Island, NY, USA) or palletized for direct use as per requirement of different sets of experiments. Plasma, wherever required, was isolated by centrifuging undiluted peripheral blood at 100g for 10 minutes prior to isolation of PBMCs and stored at -80°C in 250 µl aliquots until needed.
**Isolation of Serum:**

Serum was isolated from 67 TA patients and 50 healthy controls at different periods of times during the visit of the patients to the hospital. All serum samples of patients and controls were used for the detection of AECA to identify AECA positive and negative sera for different experimental use and for the detection of anti-annexin-V antibodies and evaluation of their relationship with AECA.

After informed consent of the subjects selected for the study 2 ml of venous blood was collected from each subject in a sterilized glass tube containing no anticoagulant and incubated at 37°C for 30 minutes to allow the blood clotting and separation of serum. The blood clots were retracted from separated serum by incubating the blood at 4°C for 15 minutes and finally the tube was centrifuged at 450g for 10 minutes to isolate the serum. The serum thus obtained was aliquoted in 500 µl volumes in sterilized micro tubes and stored at -80°C until required.

**Analysis of Cytokine Gene Repertoire of PBMCs:**

Cytokine gene repertoire including TNF-α, IFN-γ, IL-2, IL-3, IL-4, IL-10, IL-12 and GM-CSF of PBMCs under constitutive and induced conditions was analyzed by RT-PCR, which included following important steps: stimulation of PBMCs, RNA extraction, RT-PCR and semi-quantification of PCR products (amplicon), which are sequentially described as under:

**Stimulation of PBMCs:**

Twelve million of freshly isolated PBMCs were taken. One aliquot of 5 x 10^6 cells was immediately used for ribose nucleic acid (RNA) extraction
and the remaining (usually ≥7.5 x 10^6) cells were put for activation to detect constitutive and induced cytokine gene expression, respectively.

To induce cytokine gene transcription, the cells were stimulated with PHA + PMA as described previously (Sullivan et al, 2000). Briefly, the isolated cells were suspended in complete medium consisting of RPMI-1640 supplemented with 3 mM of L-glutamine, 10 mM of HEPES buffer, 1 mM of sodium pyruvate and 10% of heat inactivated fetal bovine serum (all from Gibco BRL, Grand Island, NY, USA). A total of 5 x 10^6 cells/well in a final volume of 2 ml of complete medium were dispensed in a 6 well tissue culture plate (Nunclon, Roskilde, Denmark) and incubated for 4 hours under standard tissue culture conditions in the presence of 2 µg/ml of PHA (Sigma) plus 25 ng/ml of PMA (Sigma). After incubation cells were harvested and used for RNA extraction.

In an additional activation protocol, a total of 2.5 x 10^6 PBMCs in a final volume of 1 ml of complete medium were stimulated by incubating with 10 µg/ml of lipopolysaccharide (LPS) (Sigma) for 12 hours as above to detect IL-12 gene expression in the cells (Lee et al, 1996).

**RNA Extraction:**

Total cellular RNA from unactivated and activated PBMCs was extracted by modified guanidinium thioctyanate-phenol-chloroform method as described (Gauthier et al, 1997) with some modifications.

Briefly, a pellet of 2.5-5 x 10^6 cells in a sterile 2.0 ml micro centrifuge tube (Tarson, India) was dissolved in 500µl of guanidinium thiocyanate (GT) solution (4M GT, 25 mM sodium citrate pH 7.0; 0.5% (w/v) N-lauroylsarcosine and 0.1 M 2-mercaptoethanol). Sequentially, 50µl of 2M sodium acetate (pH 4.0), 500µl of 0.1M citrate buffer-
saturated phenol (pH 4.3) and 100 µl of chloroform: isoamyl alcohol mixture (24:1) was added to the lysate with vortexing the tube after addition of each reagent. The final mixture was incubated on ice for 15 minutes and centrifuged (Biofuge fresco, Heraeus) at 10,000g for 10 minutes at 4°C. The aqueous (uppermost) phase was harvested into a fresh micro centrifuge tube and thoroughly mixed by vortexing with two volumes of 95% ethanol and centrifuged as above. The supernatant was discarded and RNA pellet was washed with 250µl of 70% ethanol. The traces of ethanol from the final RNA pellet were soaked out by inverting the tube on sterile tissue paper sheet for 1 minute and subsequent air-drying. The ethanol free RNA pellet was dissolved in 25-50µl of RNA storage solution (Ambion, Austin Tx, USA) and stored at -80°C until required.

The RNA obtained shown intact 18s and 28s bands and was free of protein and DNA as revealed by electrophoresis of the samples in 1.25% agarose gel containing 0.5 µg/ml of ethidium bromide (EtBr). The ratio of absorbances (A) at 260 and 280 nm (A<sub>260</sub>: A<sub>280</sub>) of all RNA isolates in 10 mM Tris-Cl (pH 7.5) was always >1.8 indicating a clean RNA preparation. The RNA concentration in the samples was determined by measuring the absorbance at 260 nm in an (ultraviolet) UV spectrophotometer (Beckman instruments, CA, USA). Since 1 absorbance unit at 260 nm (A<sub>260</sub> = 1) represents 40 µg of single stranded RNA per ml, the total quantity present in a sample was calculated using following formula: A<sub>260</sub> x 40 µg x sample volume in ml. Accordingly each sample was found to have 2-5 µg of total RNA.

**RT-PCR:**
Cytokine gene expression was detected by a single step RT-PCR method that includes reverse transcription of messenger RNA (mRNA) into
complementary DNA (cDNA) and its subsequent PCR amplification by a continuous thermal cycling program, using RobusT RT-PCR Kit (Finnzymes, OY, Finland) according to the manufacturer’s instructions. Briefly, for each RT-PCR reaction 50 ng of total RNA was mixed to a standard reaction mixture consisting of 1x reaction buffer, 1.5 mM MgCl₂, 200 µM of each dNTP, 10 pmol each Oligonucleotide primer, 5 unit of avian myeloblastosis virus reverse transcriptase (AMV-RT), 2 unit of DyNAzyme EXT DNA Polymerase and di-ethyl pyrocarbonate (DEPC) (Sigma) treated sterile water up to a final volume of 50 µl in a sterile 250 µl PCR tube (GENEI, Bangalore, India). The reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) and consisted of following steps: (a) an initial cDNA synthesis at 48°C for 45 minutes followed by inactivation of AMV-RT and denaturation of cDNA/RNA hybrid at 94°C for 2 minutes, (b) PCR amplification by 35 sequential cycles of denaturation (94°C for 45 sec), annealing (at cytokine specific temperature for 45 sec) and primer extension (72°C for 60 sec) and (c) a final extension at 72°C for 7 minutes followed by cooling to 12°C as end of the reaction.

Amplification of RNA for the house keeping gene β-actin was used as internal quality control. The sequences of the primers (Quiagen, Hilden, Germany), optimal PCR annealing temperatures (Ta) and size of the amplified PCR product are given in Table: 1.

A negative and a positive control were included in each set of experiments to rule out amplification of contaminating genomic DNA if any and to confirm the validity of the procedure, respectively. The negative control consisted of a reaction mixture with no AMV-RT while the positive control for each cytokine consisted of RNA derived from PHA+PMA or LPS stimulated PBMCs of a healthy individual.
Table: 1 Oligonucleotide primers and related information.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence: Forward: 5'-3'</th>
<th>Reverse: 5'-3'</th>
<th>Ta (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>GAGTGACAAGCGCTGTACGCACTGTAGCCCATGTTGTAGCG</td>
<td>GCAATGATCCTCCGACCTGACCTGCCAGACT</td>
<td>66</td>
<td>444</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TCTGCATCGTTTTGGGTTCCTC</td>
<td>TCAGCTTTTCGAAGTCATCCTC</td>
<td>55</td>
<td>321</td>
</tr>
<tr>
<td>IL-2</td>
<td>ATGTACAGGGTGCAACTCTCTTGCTTTG</td>
<td>GTTAGTGTTGAGATGAGGATTCAGTTTTCAC</td>
<td>55</td>
<td>457</td>
</tr>
<tr>
<td>IL-3</td>
<td>TCCAAACATGAGCGCCGCTGCTCCT</td>
<td>CATCAGAATGCTCTTGGCTTCTC</td>
<td>60</td>
<td>211</td>
</tr>
<tr>
<td>IL-4</td>
<td>CCTCTGTTCCTCTGCTATGCA</td>
<td>GCCGTTTCAGGAATCGGATCA</td>
<td>58</td>
<td>300</td>
</tr>
<tr>
<td>IL-10</td>
<td>ACAGCTCACCCCTGCTCTGT</td>
<td>AGTTCACTGCGCTTTGATG</td>
<td>60</td>
<td>327</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>AGTGTCAAGGAGCAGCAGGAGC</td>
<td>AACGCAATGATCAGGAGGCA</td>
<td>60</td>
<td>363</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>CAGCATGTGATAGCGCCACAGGAGG</td>
<td>CTCTGGACTGCTCCAGCAGTCA</td>
<td>60</td>
<td>390</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CACTCTCCAGCCTTCTTCTCC</td>
<td>CGGACTCGTCATACTCCCTTCTT</td>
<td>62</td>
<td>311</td>
</tr>
</tbody>
</table>

Ta = Annealing Temperature of primers.
bp = Base Pairs.

Semi-quantification of PCR Products:

Semi-quantification of cytokine gene expression was carried out by comparing the signal intensities of a cytokine PCR product to that of β-actin of the same RNA sample using agarose gel electrophoresis followed by densitometric scanning of the amplicon (product) bands as described (Walker, 1998) with some modifications.

Twelve µl aliquot of an amplicon was mixed with 2.5 µl of gel loading dye and electrophoresed through 1.5-1.8 % agarose gel (Sigma) in 0.5X TBE buffer (89 mM Tris base, pH 7.6; 89 mM Boric Acid and 2
mM EDTA) containing 0.5 µg/ml of EtBr at 80V constant voltage field until the dye front had migrated for a distance of 6 cm (usually 45 minutes). The intensities of the product bands were quantified by densitometric scanning of gels using a working station that included a UV Tran-illuminator light box and Kodak CD40 Digital Camera with 1D Gel Analysis Software connected to computer. Ratios of cytokine: β-actin densities in patients and controls were compared to analyze the difference in cytokine gene expression between the groups. A 100 base pair (bp) DNA ladder molecular marker (MBI, Fermentas, Lithuania) was run in every gel to confirm the size of the PCR product.

The intensity of each cytokine PCR product in each subject was calculated as percent of β-actin and data of each cytokine in each group was expressed as mean ± standard deviation (SD).

**Intracellular Assay of T-cell cytokines:**

Production of different pro-inflammatory cytokines viz. TNF-α, IL-2, and IFN-γ by peripheral blood T-cells was detected intracellularly by dual colour flow cytometry using PMA plus ionomycin stimulated PBMCs and staining the cells with pre-conjugated cytokine and cell surface marker mAbs following para-formaldehyde-saponin procedure as described previously (Mascher et al, 1999) with some modifications of our own.

Briefly, to induce production of cytokines and their intracellular accumulation, 2 x10^6 PBMC/ml/well taken in a 24 well plate (Costar, Corning, NY, USA) were stimulated with a combination of 50 ng/ml PMA and 1 µg/ml of ionomycin in the presence of 5 µg/ml brefeldin A (all from Sigma). The cells were incubated for 5 h at 37°C in CO₂ incubator (Sanyo, Japan) under standard tissue culture conditions. The
stimulation time of 5 h was found to be optimal on the basis of a preliminary time kinetic study performed for the detection of these cytokines. At the end of the incubation, the cells from each well were harvested in a flow cytometer tube (Becton Dickinson, Mountain View, CA, USA), washed (400g x 15 minutes) with cold phosphate buffered saline (PBS, pH 7.4) and fixed with ice-cold 1% para formaldehyde (Ranbaxy, Nagpur, Punjab, India) in PBS for 20 minutes at 4°C. After one wash as above the cells were resuspended in saponin buffer consisting of 0.1% saponin in Earl’s balanced salt solution (EBSS; Gibco) and incubated for 10 minutes at room temperature for permeabilization.

For immunoassaying of intracellular cytokines approximately 0.5 x 10^6 permeabilized cells taken in a cytometer tube and incubated for 30 minutes at room temperature in dark with optimal quantity of fluorescein isothiocyanate (FITC) conjugated mouse mAbs to human TNF-α, IL-2 and IFN-γ or matched isotype control (all from Becton Dickinson). Cells were washed with PBS containing 0.2% bovine serum albumin (SRL, Mumbai, India) and 0.1% sodium azide (SRL) and stained as above with R-phycoerythrin (PE) conjugated mouse anti-human CD3 or its isotype control (Becton Dickinson). After two washes with PBS cells were finally resuspended in 500 µl of 1% para formaldehyde containing 0.1% sodium azide, stored at 4°C and analyzed within 12 h of staining time.

The stained cells were acquired and analyzed on FACS calibur flow cytometer (Becton Dickinson) using CELLQUEST software. On the basis of typical forward and side scatter properties a gate for lymphocyte was set to exclude dead cells and contaminating monocytes from the analysis. A total of 5, 000 cells were acquired in the lymphocyte gate and TNF-α, IL-2 and IFN-γ containing T-cells in the gate were analyzed by
detection of double (anti-cytokine-FITC and anti-CD3-PE) stained cells. The percentage of these double positive cells was determined by statistical analysis of the quadrants set on the basis of used isotype controls.

The PBMCs of the patients with active TA were further stained with FITC-conjugated IL-2 or TNF-α and PE-conjugated anti-CD-4 or anti-CD-8 antibodies as described above to delineate the subsets of CD3+ T-cells producing these cytokines.

**Plasma Cytokine Assays:**

The plasma levels of TNF-α, IL-2 and IFN-γ were detected by quantitative ELISA using commercial kits (Quantikine: R & D Systems, Minneapolis, MN, USA) as per manufacturer’s instructions. The concentration of each cytokine was measured in pg/ml. The sensitivity of these kits for the detection of TNF-α, IL-2 and IFN-γ was 4.4, 7 and 8 pg/ml, respectively.

**T-cell Proliferation Assay:**

The proliferative responses of CD3+ T-cells and its CD-4 and CD8 subsets to Mycobacterial HSP-65 and human HSP-60 was evaluated by flow cytometric determination of bromodeoxyuridine (BrdU) incorporation into the DNA of proliferating cells and their phenotypes using FITC-conjugated BrdU and PE-conjugated cell surface marker mAbs (Caryon et al., 1992).

Freshly isolated PBMCs at the concentration of 1x10^6 cells/ml in a final volume of 1 ml of complete medium containing 10 µg/ml of recombinant HSP-65 or HSP-60 (LIONEX GmbH, Germany) or no any HSP antigen, were cultured in flat bottom 24 wells tissue culture plate (Nunc, Roskilde, Denmark) under standard tissue culture conditions for 72
During last 24 h the cells were pulsed with 30 µg/ml of BrdU to allow its incorporation into DNA of the proliferating cells.

Following the incorporation of Bromodeoxyuridine (BrdU), cells were harvested directly in FACS tubes, washed once with PBS and fixed with 1% (w/v) para-formaldehyde for 30 minutes at room temperature (RT) followed by their permeabilization with 0.1% saponin for 15 min at RT. The permeabilized cells were treated with 50 Kunitz U/ml DNase-I (Activity 400-600 KU/ml protein; Sigma) for 10 min at 37°C to induce DNA denaturation. Following washing with PBS, cell pellet was suspended in 10 µl of PBS containing 2% bovine serum albumin (BSA) (staining buffer) and stained with saturating concentration of FITC-conjugated antibody to BrdU (Becton Dickinson, Mountain View, CA, USA) at RT in dark for 45 min. The cells were washed with staining buffer and split equally in three different fluorescence activated cell sorter (FACS) tubes and stained as above with PE-conjugated CD3, CD4 and CD8 mAbs (Becton Dickinson). After two washes with PBS cells were finally resuspended in 500 µl of 1% para formaldehyde containing 0.1% sodium azide, stored at 4°C and analyzed in flow cytometer (FACScaliber, Becton Dickinson) using Cell-Quest software.

The PBMCs of at least two patients with active tuberculosis and those of one healthy individual were included in each set of experiments as positive and negative controls, respectively. A total of 10,000 cells from each tube were acquired in the lymphocyte gate and proliferating T-cells were identified as double positive cells stained with BrdU and CD3+, CD4+ or CD8+ mAbs in the quadrants. The cut-off value for T-cells proliferative response was taken as mean + 2 SD of the percentage T-cell proliferation observed in healthy controls.
**Isolation and Culture of Human Endothelial Cells:**

Human ECs were isolated from human umbilical cords by collagenase digestion of endothelial lining of the cord veins as described previously (Jafee et al, 1973).

Briefly, umbilical cords of fresh deliveries (>24 hours old) were aseptically collected in PBS containing bacteriostatic levels of antibiotics. Vein lumen of the cord is washed with PBS and perfused with 0.05% (w/v; PBS) solution of collagenase (Gibco BRL) and the cord was incubated at 37°C for 20 minutes to allow digestion of endothelial lining of the cord vein. The collagenase solution containing detached endothelial cells was collected in a sterile 50 ml conical centrifuge tube (Tarson, India). The cells were palletized by centrifugation at 400g for 10 minutes and suspended homogeneously in 5 ml of culture medium consisting of medium-199 containing 20% heat inactivated FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin (all from Gibco BRL). The cell suspension was put into a 25-cm² tissue culture flask (Tarson) precoated with 1% (w/v; PBS) gelatin (Sigma) and the flask was incubated for overnight in a humidified CO₂ incubator (Sanyo, Japan) under standard tissue culture conditions.

On the following day the flask was washed twice with PBS to remove any non-adherent cells /debris and 5 ml of fresh culture medium was added and again incubated in CO₂ incubator until a semi-confluent monolayer of endothelial cells is obtained (usually 24-48 hours). These primary endothelial cultures were used in different experiments.
Detection of AECA:

To use the AECA positive and negative sera in different experiments of the study, different isotypes of these antibodies were detected in the stored sera of the patients and controls by an endogenously developed C-ELISA using fixed human endothelial cells as antigens according to the previously described method (Nityanand et al, 1997b).

Briefly, primary semi-confluent cultures of endothelial cells were harvested from tissue culture flak by treating the cells with 0.05% (w/v; PBS)) trypsin and 0.02% (w/v; PBS) of EDTA (Gibco BRL). The cells were palletized by centrifugation (400gx 10 minutes) resuspended in a optimal volume of culture medium so as to make a final concentration of 0.2 x 10^6 cell/ ml. Two hundred microliter of this cell suspension (4 x 10^4 cells) were seeded in each well of a 96 well flat bottom tissue culture plate (NUNC Roskilde, Denmark) pre-coated with 1% gelatin. The cells were allowed to grow for 24 hours to get a semi-confluent monolayer of endothelial cells in each well of the plate. After two washes with PBS, the monolayer of cells was immobilized to solid phase by fixing with 0.2% of glutaraldehyde (SRL, Mumbai, India) for 20 minutes at room temperature. Washing between the steps was performed with phosphate buffered saline (PBS, pH 7.4) containing 0.2% bovine serum albumin (wash buffer). Non-specific binding sites were blocked by incubating the plate with 2% bovine serum albumin in PBS for 1 hour at 37°C. Test and reference sera were diluted 1:100 with wash buffer or wash buffer alone were added 100 µl/well in triplicates and the plate was incubated for 2 hours at 37°C. The bound antibodies were detected by incubating the plate with 100 µl /well of alkaline phosphatase conjugated anti-human IgG (Fab\textsubscript{2} specific; 1: 5000), IgM (µ Chain Specific; 1:5000) or IgA (α-Chain specific; 1: 5000) (all from Sigma, St Louis, MO, USA). The colour reaction was developed by
adding 100µl/well of p-nitrophenyl phosphate (1 mg/ml, Sigma) and the absorbance was read in an automated ELISA reader (Tecan Spectra, Austria) at 405 nm.

The cut off value for stating that a sample is AECA positive was taken as mean + 2SD of the optical densities (ODs) of the 50 controls samples run in parallel. The intra- and inter- assay coefficient of variation were <5% and 15%, respectively.

**Complement and Cell Mediated Cytotoxicity of AECA:**

Complement dependent cytotoxicity (CDC) of AECA to untreated and cytokine stimulated endothelial cells was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Fujieda et al, 1997) with some modifications. Antibody dependent cellular cytotoxicity (ADCC) of AECA to endothelial cells was carried out by 51Cr release assay (Savage et al, 1991).

**CDC of AECA:**

Primary culture ECs were seeded in 96 well flat bottom tissue culture plates (Nunc) at a concentration of 4x10⁴ cells per well and cultured overnight. Test sera were diluted 1:10 in plain MEM (Gibco BRL) medium, heat inactivated (56°C for 30 minutes) and sterilized by filtration through 0.22µm membrane filters. Hundred µl of each sample was added in triplicate wells. The positive control was 1:10 diluted murine anti-human monoclonal antibody W6/32 (DAKO, Glostrup, Denmark) which is IgG2a isotype directed against HLA class 1 monomorphic determinants and causes 34 to 57% ECs lysis in presence of the complement (17). This monoclonal antibody also served to check reproducibility of the assay. The plate was incubated at 37°C for 3 hrs in CO₂ incubator. After 2 washes with plain MEM medium, 100 µl of freshly thawed human AB serum
diluted 1:4 with plain MEM medium was added to each well as a source of complement. Hundred µl/well of 3% Triton X-100 and heat inactivated same human AB serum (HABS) were added in two set of triplicate wells to measure MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] uptake by lysed cells (background uptake) or maximum uptake of MTT, respectively. The plate was again incubated as above for 2 hrs. After 2 washes, 100 µl of 1:4 diluted MTT (Sigma St Louis MO) solution (5mg/ml) was added in all the wells and the plate was incubated at 37°C for 4 hrs as above. MTT solution was removed and 100 µl of dimethyl sulfoxide (Sigma) was added in all the wells. The plate was incubated at 37°C for 30 minutes with intermittent shaking to solubilize the formazan crystals. The plate was read at 570 nm in an ELISA reader (Tecan Spectra, Austria). The percentage cytotoxicity for each sample was calculated using the following formula:

\[
\text{% Cytotoxicity} = \frac{\text{Maximum uptake} - \text{Sample uptake}}{\text{Maximum uptake} - \text{Background uptake}} \times 100
\]

The cut off value of the cytotoxicity of test sera was taken as mean + 2 SD of the cytotoxicity of AECA negative TA sera.

**CDC of AECA to IL1-β and TNF-α treated Endothelial Cells:**

AECA positive sera, which were found to be cytotoxic to untreated (resting) ECs, were further evaluated for cytotoxicity after cytokine pre-treatment of the targets. For this purpose the overnight grown ECs were treated with 10 U/ml of IL1β or 100 U/ml of TNFα (both from R&D
Subjects & methods

Systems, Minneapolis, MN, USA) for 6 hours at 37°C and the cytotoxicity experiments were carried out as described above.

**ADCC of AECA:**

To detect ADCC of AECA, the primary culture ECs were seeded in 96 well flat bottom tissue culture plates (Nunc) at a concentration of 2 x 10^4 cells/well and cultured overnight. The ECs were labeled with 5 μCi of 51Cr (Sodium chromate, 1mCi/ml, BARC, Bombay, India) for 4 hrs at 37°C in CO_2 incubator. While this incubation was going on PBMCs from a single donor were isolated as described earlier, suspended in complete medium at the concentration of 1x 10^6 cells/ml and incubated in a 25 cm^2 tissue culture flask at 37°C in CO_2 incubator for 2 hrs to deplete adherent cells. The non-adherent cells were harvested from the flask and re-suspended in RPMI-1640 with 10% heat inactivated FBS (complete medium) at a concentration of 10 x 10^6 cells/ml. These cells were used as effectors in the cytotoxicity assay.

After optimal labeling of the targets, the excess of ^{51}Cr was removed and the plate was washed three times with plain MEM medium with 30 minutes dwelling time in each wash. Test sera were diluted 1:2, 1:5, 1:10, 1:25, and 1:50 with MEM medium containing 10% FBS (Gibco BRL). The diluted sera were then heat inactivated and sterilized by membrane filtration. Hundred μl/well of MEM medium with 10% FBS (Sigma) or diluted AECA positive or negative sera of TA patients were added in the plate in triplicates. The plate was incubated for 2 hours at 37°C in CO_2 incubator. After two washes with plain MEM medium, effectors were added in each test well in a final volume of 200 μl/well of C-RPMI at an effector: target (E: T) ratio of 10:1, 25:1 50:1 and 100:1. One set of triplicate wells contained 200 μl of 3% Triton X-100 (SRL) and another set of triplicate wells contained 200 μl/well of medium RPMI-1640.
with 10% FBS to determine maximum and spontaneous release of radioactivity, respectively. Two AECA positive scleroderma sera producing 25-30% and 40-45% of cytotoxicity, respectively, at E: T ratio of 25:1, were used as positive controls in each run to check the reproducibility and validity of the assay. After centrifugation (250 x g, 5 minutes) the plate was incubated as above for 6 hrs. The plate was again centrifuged at 250 x g for 5 minutes and 100 µl of cell free supernatant was collected from each well and released radioactivity was measured using a multigamma counter (LKB, Pharmacia, Sweden).

The percentage cytotoxicity for each sample was calculated using the following formula:

\[
\text{Sample release - spontaneous release} \\
\% \text{Cytotoxicity} = \frac{\text{Sample release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100
\]

The cut off value of the cytotoxicity of test sera was taken as mean + 2 SD of the cytotoxicity of AECA negative TA sera.

**Characterization of Antigenic Targets of AECA:**

Characterization of antigenic targets of AECA was carried out by immunoblotting using whole cell lysate of ECs and include following main steps: Preparation of EC lysate, protein estimation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.
Preparation of EC Lysate:
The whole cell lysate of human ECs was prepared by a standard protocol (Sambrook et al., 1989). The primary human ECs were isolated from human umbilical cord veins, grown in 25 cm² tissue culture flasks and confluent cultures were harvested from the flasks by trypsinization as described above earlier. The cells were collected in 50 ml centrifuge tubes (Tarson) and washed twice with cold PBS at 400g for 10 minutes. To a pellet of $10 \times 10^6$ cells (or simply 5 volumes of the cell pellet), hot (85°C) 1x SDS-PAGE sample buffer without dye and 2-mercaptoethanol [100mM Tris-Cl (pH 6.8), 8% (w/v) SDS and 20% (v/v) glycerol] was added to lyse the cells. Immediately, the tube was placed in a boiling water bath (100°C) for 10 minutes to inactivate the proteases. The lysed cells were sonicated on ice for 10 times at 25% duty cycle of 10 seconds each (Branson Sonifier, Panbury, CON) and centrifuged at 10,000g for 10 minutes at 4°C (Kubota, High Speed Centrifuge, Tokyo Japan) to pelletize the nuclear contents mainly the chromosomal DNA. The final supernatant thus obtained was harvested, aliquoted and stored at −80°C until analysis.

Protein Estimation:
The protein contents in the EC lysate were estimated by modified Lowry’s method (Markwell et al., 1978) with bovine serum albumin (BSA) (SRL, Mumbai, India) as standard.

Briefly, 100 parts of reagent-A (2% sodium carbonate, 0.4% sodium hydroxide, 0.15 sodium tartarate and 1% SDS) were mixed with 1 part of reagent-B (copper sulfate). A sample volume of 1 ml of different dilutions of the cell lysate was added to 3 ml of above reagent mixture and incubated for 15 minutes at room temperature (RT). Following incubation the samples were mixed with 300 µl of 1N Folin-Ciocalteu’s reagent (SPECTROCHEM, Mumbai, India) and incubated for 45 minutes at RT in
dark. The ODs of the different samples were read in spectrophotometer (Sanyo, Tokyo, Japan) at 660 nm. The protein concentration in each sample was calculated with the help of calibration curve drawn by determining the OD values of known concentrations of BSA as above. The concentration of the protein in the original cell lysate was finally adjusted to 500 µg/ml with the 1x SDS-PAGE sample buffer.

**SDS-PAGE and Immunoblotting:**

SDS-PAGE of the EC lysate was carried out under reducing conditions as described (Laemmli, 1971) with minor modifications. Briefly, 500 µl of EC lysate containing approximately 250 µg of total cellular proteins was mixed with optimal quantity of bromophenol blue dye and 2-mercaptoethanol was subjected to 12% SDS-PAGE for 2 hours at 250V/25 mA in a medium size gel apparatus (Bio-Rad, Richmond, CA, USA) until the tracking dye reached the bottom of the gel. Pre-stained low molecular weight (MW) standards ranging from 29.7 to 142.9 kDa in size (Bio-Rad) were included in each run.

Separated proteins from the gels were electro-transferred onto the transfer buffer pre-equilibrated nitrocellulose sheets (0.22µ, mdi, Advanced Microdevices, Amballa Cantt, India) by semidy blotter (ATTO Corpn, Tokyo, Japan) at 40 mA/10V for 1 hour as described (Sambrook et al, 1989). The equivalent loading and transfer in each run was confirmed by staining the blots with Ponceau-S (Sigma). The blots were blocked in 10 mM Tris-buffered saline (TBS) (10mM Tris Base and 150 mM NaCl; pH 7.5) containing 5% (w/v) BSA (SRL) and 0.2% (v/v) tween-20 (SRL) for 3 hours at RT and cut into stripes of defined dimensions. These blot stripes were incubated with patient or control sera (as source of AECA) diluted 1:50 in TBS containing 1% BSA and 0.1% Tween-20 (sample diluent) for 2 hours at RT. The AECA reactivity to endothelial proteins was detected
Subjects & methods

by incubating the stripes for 2 hours at RT with rabbit monoclonal anti-
human IgG-HRP (DAKO, Carpinteria, CA, USA) diluted 1:2000 in the
sample diluent followed by colour development using diaminobenzidine-
hydrogen peroxide chromogenic system (DAKO) as substrate. Each
incubation was followed by 6 washes of 5 minutes each with wash buffer
(TBS containing 0.1% Tween-20) and all incubations and washes in the
procedure were carried out on a rocking shaker (Lead, Bangalore, India).

The molecular weight (MW) of the bands of immunoreactivity on
each strip was determined from standard curve plotted using log MW
versus relative mobility of the MW of the standards used in the procedure.

Detection of AA5A:

The AECA pre-screened sera were investigated for the presence of AA5A,
which were detected by ELISA using recombinant human annexin-V as
antigen according to a validated procedure described previously

Briefly, gamma-irradiated 96 well flat-bottomed plates (Nunc,
Roskilde, Denmark) were coated with 0.5 µg/well of purified recombinant
annexin-V (BD Phammingen, Mountain View, CA) in a final volume of
100 µl of carbonate- bicarbonate buffer (0.05M,pH 9.6) and incubated at
4°C for 24 hours. Following this and each of the subsequent incubation
steps washing was performed with PBS (pH 7.4) containing 0.2% BSA and
0.05% Tween-20 (Wash buffer). The non-specific binding sites of each
well were blocked by adding 250 µl of PBS containing 3% BSA and
incubating the plate at 37°C for 2 hours. Test and reference sera diluted
1:300 with wash buffer, were added 100 µl/well in duplicates with one set
of duplicate wells containing wash buffer alone (antibody blank) and the
plates were incubated for 2 hours at 37°C. The bound antibodies were
detected by incubating 100 µl/well of 1:3000 diluted γ chain specific goat
anti-human IgG-alkaline phosphatase (Sigma, St Louis, MO, USA). The colour reaction was developed by adding 100 µl/well of 1mg/ml of p-nitrophenyl phosphate (Sigma) in di-ethanolamine buffer (pH 9.8) and the absorbance was read in an automated ELISA reader (Tecan Spectra, Austria) at 405 nm.

Two positive and negative sera were included as standards in each run. Each plate was read when the ODs of positive controls reached 1.0-1.2 (always within 35 minutes). The blank value of the plate was subtracted from the sample value to get specific OD for each sample. The cut off value for determining a sample to be positive, was taken as mean + 2 SD of the ODs of the normal controls. The inter-assay coefficient of variation was 8.5%.

**Evaluation of Relationship of AA5A and AECA:**

To evaluate a relationship between AA5A and AECA, AECA were studied in AA5A positive sera of the patients by C-ELISA using fixed ECs as antigens as described above earlier.

**Statistical Analysis:**

Data were analyzed using Z-statistics, Mann-Whitney U-test or Student’s t-test for independent samples where appropriate and a p-value of <0.05 was considered to be statistically significant.