Original article

Analysis of the clinical profile, autoimmune phenomena and T cell subsets (CD4 and CD8) in Takayasu's arteritis: A hospital-based study

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ABSTRACT

Objective. *To evaluate clinical and immunological abnormalities in patients with Takayasu's arteritis (TA) from India, with particular reference to autoimmune perturbations and abnormalities in T cell subsets (CD4 and CD8 cells).*

Methods. *16 consecutive patients with TA (11 females and 5 males) underwent clinical and laboratory evaluation inclusive of flow cytometric analysis of T cell subsets (CD4 and CD8). A control population of 94 age- and sex-matched blood donors was used to determine the normal T cell subsets. Student's t-test was used to compare the means.*

Results. *The mean age at onset was 23.4 + 2.3 yrs. Common symptoms observed were headache, limb claudication, abdominal pain and visual disturbance/blackout. Common clinical signs observed included reduced arterial pulsations, bruits, and a BP difference > 10 mm Hg in the upper limbs. Systemic hypertension was documented in 12 patients. The mean absolute lymphocyte count in the patients was 2289/mm^3. The mean CD4 count and CD4% were 1003 and 41 respectively; the mean CD8 count and CD8% were 755 and 34, respectively; and the mean CD4/8 ratio was 1.41. The patients had statistically significantly higher CD8 but not CD4 T cell values than controls. IgG and IgM immunoglobulin levels were increased. The mean multitest CMI score in patients using CMI multi-test device of Pasteur Merieux was 14.6 mm. Two patients had an anergic response, 4 a partial response (1 – 13 mm), and 6 a full response of >13 mm. Four patients hyper-responded with a score of >20 mm. ANCA was positive in 2 patients. ANAwas positive in 3 patients. IgG anticardiolipin was positive in 12 patients and IgM in 3; overall 12 patients were anticardiolipin positive by ELISA. Anti-β2GPI of the IgG variety was found to be positive in 3 patients and IgM in 2 patients; overall 3 patients being positive for the same. Nine of the patients with active disease were started on a combination of moderate dose prednisolone (20 – 40 mg once daily) along with weekly oral methotrexate (7.5 – 15.0 mg). Surgical intervention was required in 6 patients.*

Conclusion. *This study found an increase in CD8 positive T cell subsets, increased IgG and IgM immunoglobulin levels, and the presence of autoantibodies including ANA, ANCA, anticardiolipin and anti-β2GPI antibodies in TA patients. TA may be an autoimmune disorder with T cell aberrations. The relationship with antiphospholipid antibodies and anti-β2GPI needs to be explored and confirmed by other larger studies. The strikingly positive responses to tuberculin, as well as the multitest CMI also indicate exaggerated T cell responses and cell mediated immunity in Takayasu's arteritis. Immunosuppressive therapy was successful in controlling disease activity in the majority, but surgery was needed for irreversible stenotic lesions.*

Introduction

Takayasu's arteritis (TA) is a chronic panarteritis primarily affecting young women. It involves the aorta, its main branches, and the pulmonary and coronary arteries. It is characterized by granulomatous changes in the media and adventitia of the involved vessels with progression to sclerosis over time causing steno-occlusive lesions and aneurysmal dilatations (1) Though this entity is seen more frequently in

Asians, it still remains uncommon in the breakup of patients seen in the rheumatology OPD of our large tertiary referral center in Pune, Western India. Thus, our hospital has recorded only 45 cases of TA over five consecutive years (2). However, data on immune aberrations in TA from this part of the world is scant. Hence, this study was undertaken to evaluate patients with Takayasu's aortoarteritis, with special emphasis on selected immunological parameters including enumeration of the T cell subsets (CD4 and CD8) by 2-color flow cytometry.

Materials and methods

16 consecutive patients (11 females and 5 males) fulfilling the ACR criteria for Takayasu's arteritis (TA) (3) were studied in the Rheumatology and Clinical Immunology Centre of our hospital. Using a standardized data extraction proforma, a detailed history and epidemiological data was taken. A thorough clinical examination was carried out and the findings were recorded.

Flow cytometric analysis of T cell subsets (EPICS-XL, Coulter) was done for all patients as per the methodology used by us in a recently published study to establish normal values for absolute lymphocyte counts, CD4 counts and percentages, CD8 counts and percentages, and the CD4/8 ratios in 94 healthy volunteer blood donors (4). Blood specimens were collected by venipuncture and anticoagulated with ethylenediamine tetra acidic acid (EDTA, 2 mg/ml). Anticoagulated blood samples were tested within 1 hour of collection.

For each patient sample, two 12 x 75 mm test tubes were labeled for monoclonal antibody tests and appropriate isotypic controls, respectively. 100 µl of anticoagulated blood was pipetted into the bottom of each properly labeled test tube ensuring that the inside surface and top of the tube was free of blood. Next, 10 µl of CYTO-STAT/ Coulter Clone T4-RDI/T8-FITC and 10 µl of idiotypic control (CYTO-STAT/CoulterClone) were added to the test and control tubes respectively, which were vortexed and incubated for 10 min at room temperature. Next, the tubes were placed in the Coulter Q-PREP Workstation and the 35 seconds cycle initiated so as to lyse the RBCs with ImmunoPrep A (formic acid 1.2 ml/l), stabilise the leucocytes with ImmunoPrep B (sodium carbonate 6 g/l) and fix the cell membranes with ImmunoPrep C (paraformaldehyde 10 g/l).

Then the prepared cells were analysed on a Coulter EPICS-XLflow cytometer using dual fluorescence analysis, properly standardized (5) and gated on lymphocytes. Briefly, this consisted of collecting a 90º LS vs FALS histogram, gating on the lymphocyte population, collecting log-integrated green and red fluorescence (LGFL+ LIRFL) gated on lymphocytes of 90º LS vs FALS, then determining the percentage of positively stained cells by integrating a clear fluorescence peak (single color) and also by obtaining quadrant statistics (dual color). CYTO-STAT/Coulter Clone T4-RDI/T8-FITC/T3-PE reagent was used on one sample in each run to perform a 3-color analysis and cross-check the accuracy of the 2-color procedure. A positive control in the form of COULTER CYTO-TROL control cells (lyophilized lymphocytes with a known quantity of CD4 and CD8 surface antigens) was also used to cross-check results. Total white blood cell (WBC) counts, absolute lymphocyte counts (ALC) and also hemoglobin (Hb) values were determined on a Coulter machine (AcTDiff), and then absolute values of CD4 and CD8 cells were calculated by multiplying the patient's ALC with the % of the particular T-cell subset obtained by flow cytometry.

Student's t-test was used to compare the means of the parameters obtained in the present study with the control values obtained in the normal blood donor study. Other immunological evaluation for these patients included: immunoglobulin assays; estimation of ANA, IIF and ELISA for anti-MPO and anti-proteinase 3 ANCA; IgG and IgM β2 glycoprotein I (β2GPI) estimation by ELISA; and lupus anticoagulant assessment by APTT.

Multi-test cell mediated immunity skin testing was done using the "multi-test CMI" device by PASTEUR MERIEUX, Lyon, France. This was a disposable plastic applicator consisting of eight sterile heads pre-loaded with 7 delayed hypersensitivity skin test antigens and a glycerine negative control. The 7 antigens used were tuberculin, candida, streptococcus, proteus, tetanus, diphtheria and trichophyton. An induration of 2 mm or more for any of the antigens was considered to be normal reaction and a consolidated score was calculated for each patient. Patients with a score of zero were considered as anergic, those with a score of 0 to 13 mm as partial responders, and those with 14 mm or more as full responders. Additionally, tuberculin skin testing was done by injecting 10 TU of PPD intradermally and the indurations were read by palpation in 2 directions (vertically and horizontally) after 72 hours, with the mean values in mm being taken as the final reading. Angiographic and doppler evaluation of the blood vessels and echocardiographic evaluation of the heart was also done in all cases.

Results

Clinical features

The mean age at onset was 23.4 + 2.3 yr. The female to male ratio was 3.7 to 1. The median delay to diagnosis was 3 years and 6 months. The first appreciable symptom in the patients studied, details of the subsequent symptomatology that developed, the clinical signs observed, and complications encountered are given in Table I.

Disease classification, complications and surgical interventions

The angiographic classification (6) was type I in 5 patients, type II in 4 patients and type III in 7 patients. The disease classification was type I (uncomplicated) in 4 patients, type II (single complication) in 9 patients, and type III (2 or more complications) in 3 patients. The number of patients who fulfilled each of the ACR criteria (3) was as follows: age < 40 years at onset in all, limb claudication in 12, reduced major pulse in 10, BP difference >10 mm Hg in the upper limbs in 9 and bruit in 9, and abnormal angiogram in all. The total number of ACR criteria satisfied was 4 in 6 patients, 5 in 4 patients and 6 in 6

Table I. Salient clinical features of 16 patients of Takayasu's arteritis.

Feature	No. of pts.	Percentage
First appreciable symptom		
Headache	5	31.3
Arm claudication	4	25.0
Pain abdomen	2	12.5
Visual disturbance/Blackout	2	12.5
Weight loss	1	6.3
Chest pain	1	6.3
Diarrhea	1	6.3
Symptomatology		
Fatigue	9	56.3
Weight loss	9	56.3
Fever	9	56.3
Malaise	10	62.5
Anorexia	8	50.0
Night sweats	2	12.5
Arthralgias	6	37.5
Vertigo	4	25.0
Syncope	6	37.5
Headache	10	62.5
Convulsions	3	18.8
Visual disturbances	6	37.5
Neck pain	5	31.3
Upper limb claudication	8	50.0
Lower limb claudication	6	37.5
Palpitations	4	25.0
Dyspnea	4	25.0
Hair loss	6	37.5
Clinical signs		
Reduced right carotid pulse	6	37.5
Reduced left carotid pulse	2	12.5
Reduced right brachial pulse	2	12.5
Reduced left brachial pulse	6	37.5
Reduced right femoral pulse	6	37.5
Reduced left femoral pulse	5	31.3
Right carotid bruit	2	12.5
Left carotid bruit	2	12.5
Right subclavian bruit	3	18.8
Left subclavian bruit	4	25.0
Abdominal bruit	5	31.3
BPdifference >10 mm Hg (upper limbs)	10	62.5
Complications		
Systemic hypertension	12	75.0
Aortic regurgitation	3	18.8
Pulmonary arterial hypertension	1	6.3
Aneurysm formation	1	6.3
Congestive cardiac failure	1	6.3
Stroke	2	12.5
Gastrointestinal hemorrhage	1	6.3

patients. Complications included: hypertension in 12 patients, aortic regurgitation in 3 patients, and aneurysm formation in 1 patient. Takayasu retinopathy was not seen in any patient. The total number of complications (Table I) was nil in 3 patients, 1 in 8 patients, and 2 in 5 patients. Operations performed included renal angioplasty in 2, aorto-bicarotid bypass in 1, carotid axillary bypass with resection anastomosis in 1, renal autotransplantaion in 1, and rt subclavian angioplasty in 1.

Table II. Results of Tcell subsetting in patient (n = 16) and control (n = 94) groups.

	Mean		95% CI		Pvalue
	Controls	Patients	Normal	Patients	
ALC (cells/mm^3)	2114	2289	1115 – 4009	1149 – 4560	> 0.05
CD4 (cells/mm^3)	865	1003	430 – 1740	474 – 2122	> 0.05
CD4%	40.18	40.72	30.75 – 49.60	29.70 – 51.74	> 0.05
CD8 (cells/mm^3)	552	755	218 – 1396	324 – 1762	< 0.05
CD8%	31.29	34.34	20.06 – 42.52 (Var = 31.526)	26.76 – 41.92 (Var = 14.360)	< 0.05
CD4/8 ratio	1.7	1.4	0.39 – 3.02	0.31 – 2.50	> 0.05

CI: Confidence intervals; ALC: absolute lymphocyte count; CD4: CD4 positive T lymphocytes; CD8: CD8 positive Tlymphocytes; CD4/8 ratio: ratio of CD4 to CD8 positive Tlymphocytes.
Significant difference in the variances of CD8% values in patient and control populations was also observed by Fisher's test (F = 2.195, P< 0.05).

Laboratory parameters

T cell subsetting. The results of T cell subsetting in the patient and control groups are given in Table II. In the patient group, mean absolute lymphocyte counts (ALC) were 2289 cells/mm^3, mean CD4 counts and CD4% were 1,003 cells/mm^3 and 40.72% respectively, and the mean CD8 count and CD8% were 755 cells/mm^3 and 34.34% respectively. The mean CD4/8 ratio was 1.4; in particular, it was <1 in 2 patients, 1 – 2 in 11 patients and > 2 in 3 patients. The values of the CD8 cell counts and CD8% were significantly higher in the patient group compared to the control population (p< 0.05). Significant difference in the variances of CD8% values in the patient and control populations was also observed by Fisher's test (F=2.195, P<0.05). There was no statistically significant difference in the other parameters such as ALC, the CD4 counts and CD4%.

Serum proteins, immunoglobulins and autoantibodies (Table III). Serum IgG and IgM levels were found to be elevated. The mean IgG was 1,673 mg/dl, IgM 229 mg/dl, and IgA 279 mg/dl. ANAwas positive in 3 patients. ANCA was positive in 2 patients. IgG anticardiolipin was positive in 12 patients and IgM in 3, overall 12 patients being anticardiolipin positive by ELISA. Anti-β2GPI of the IgG variety was found to be positive in 3 patients and IgM in 2 patients; overall 3 patients were positive for the same. Lupus anticoagulant was found to be positive in 2 patients.

Multi-test CMI and tuberculin skin test - ing. The multi-test CMI scores using the CMI multi-test device were: 14.6 (mean), with 2 patients showing an anergic response, 4 measuring 1-13 mm (partial responders), and 6 patients > 13 (responders) [in 4 patients it was > 20 mm (hyper-responders)]. The Mantoux reaction was anergic in 1 patient, 1 – 9 mm in 1 patient (partial responder), and > 13 in 6 patients (good responders) (in 3/13 it was over 15 mm).

Other investigations. Mean hemoglobin was 112 g/L. Leucocytosis was seen in only 1 patient. Raised ESR at onset was present in 8 patients and positive CRP (>1.2 mg/dl) in 3. Abnormal ECG and chest X-ray were found in 5 patients each. Echocardiogram was abnormal in 6 patients.

Treatment

After the collection of samples for laboratory analysis, 9 of the 16 patients thought to have active disease were started on a combination of moderate dose prednisolone (20 – 40 mg once daily) along with weekly oral methotrexate (7.5 – 15.0 mg). A pulse-pressure-bruit-tenderness diagram was made for each patient and this, along with the ESR/CRP values, was used as a guide to assess disease activity and response to therapy. Steroids were tapered in the majority, but the dose had

Table III. Results of other laboratory investigations in 16 patients with Takayasu's arteritis.

Laboratory investigation	Normal range	Mean value in patients
Total protein	55–80 g/L	68 g/L
Serum albumin	35–55 g/L	**29 g/L** (low)
Serum globulins	20–35 g/L	**39 g/L** (high)
Serum albumin:globulin ratio	> 1	**0.77** (low)
Serum IgG	8.0–15.0 g/L	**16.7 g/L** (high)
Serum IgM	0.45–1.5 g/L	**2.3 g/L** (high)
Serum IgA	0.9–3.2 g/L	2.8 g/L
Antinuclear antibody (ANA)	Negative at 1:40 dilution	**Positive in 3 patients** (at 1:160 dilution in one and 1:320 dilution in two)
Antineutrophil cytoplasmic antibody, perinuclear (P-ANCA)		
Qualitative by IIF	Negative	Negative
Quantitative by anti–proteinase 3 ELISA	< 4 IU/ml	< 2.8 kU/L
Antineutrophil cytoplasmic antibody, cytoplasmic (C–ANCA)		
Qualitative by IIF	Negative	**Positive in 2 patients**
Quantitative by anti–myelo-peroxidase ELISA	< 4 IU/ml	(24 IU/ml and 32 IU/ml)
Anticardiolipin antibody		
IgG	< 15 GPU	**High values in 12 patients** (mean 26.5 GPU)
IgM	< 10 MPU	**High values in 3 patients** (mean 19.8 MPU)
Anti-β2 Glycoprotein-1 antibody		
IgG	< 15 IU/ml	**High values in 3 patients** (mean 28.3 IU/ml)
IgM	< 15 IU/ml	**High values in 2 patients** (mean 29.4 IU/ml)
Lupus anticoagulant (prolonged APTTwith failure to correct with normal platelet-poor plasma)	APTTnot more than 3 seconds than control value	**Positive in 2 patients** (APTT22 secs vs 12 secs in control and APTT18 secs vs 13 secs in control respectively)

Abnormal findings are indicated in bold type.

to be stepped up again due to renewed disease activity in 2 or 3 patients. Surgical intervention was required in 6 patients, as mentioned above.

Discussion

Clinical aspects

That TA affects mainly females under the age of 40 (3) is well borne out by our study, with 11 of the 16 patients being female and the mean age at onset being 23.4 years. The diverse and non-specific features of this disease in the early "pre-pulseless" phase generally make the diagnosis difficult to suspect and this explains the median delay to diagnosis of 3.5 years in our study (1). Hypertension is seen in over 50% of patients, being documented in 75% of our cases. Vascular bruits, frequent in our study, are detected in over 80% patients (1). That claudication occurs in 30-70% patients (6, 7) is also confirmed by this study.

Laboratory aspects and immunology

Raised ESR and C reactive proteins are conventionally used to assess disease activity in these patients, with an ESR > 50 mm/hr having a 57% sensitivity and 37% specificity. However, it is disconcerting to note that surgical bypass biopsy specimens from clinically inactive patients in one study showed histologically active disease in 44% of patients (1). Thus, current laboratory markers of disease activity are insufficiently reliable to guide management. Raised ESR at diagnosis was present in only half our cases and elevated CRPin only 3.

T cell subsetting

A Medline search on the peripheral blood T cell abnormalities in TA elicits very scant data. It is reported that most of the peripheral T cells are CD4 positive Th cells or CD8 positive cytotoxic cells (8) but that overall the numbers of CD4 cells are normal (9). However, we have found significantly increased CD8 positive T cell counts and percentages ($p < 0.05$) in our TA patients as compared to the control population. Marked infiltration of the arterial wall in TA with CD8 positive lymphocytes, but not CD4 positive lymphocytes has been documented in an earlier study and argues in favor of a pathogenetic role for cytotoxic T lymphocytes (10). Therefore, cytotoxic cellular immunological mechanisms could play an important role in the pathogenesis of TA, possibly through the direct action of CD8 Tcells on large elastic arteries.

Serum proteins, immunoglobulins and autoantibodies

This study found increased IgG and IgM immunoglobulin levels, and the presence of autoantibodies including ANA, ANCA, anticardiolipin and anti-β2GPI, suggesting that TA may be an autoimmune disorder with autoantibody production. Antinuclear antibodies have not been found in the studies published thus far, and the cause of this discrepancy is unclear. On the other hand, raised antiphospholipid antibodies have been reported in some publications (11-14). Contrarily, a recent study from Mexico did not find antiphospholipid or other autoanibodies (15). The relationship with antiphospholipid antibodies and β2GPI antibodies thus needs to be explored in larger studies. ANCA positivity was reported in one of 4 cases of TA studied in 1995 (16), but could not be found in any of the 16 cases evaluated in an earlier study from 1993 (17).

Positive responses to tuberculin in 81% of 44 cases studied have been reported in a Mexican study. (18) A heightened immune response to *Mycobacterium tuberculosis* antigens, in particular to its 65 kDa HSP, have also been demonstrated subsequently (19, 20). However, no previous studies have reported on Multi-test CMI skin scores in TA patients. The strikingly positive re-

sponses to PPD as well as to multi-test CMI skin tests in our patients with no evidence of past or present tuberculosis indicates exaggerated T cell responses and cell mediated immunity, not only against tuberculin, but against all recall antigens in general. This could be an important observation to get away from the tubercular aetiology of TA, which continues to evoke interest in the literature (21).

Treatment

TA has been empirically treated with many drugs [anti-coagulants, salicylates, antimalarials, antimicrobials (especially anti-tubercular), and cytotoxics], but with the exception of glucocorticoids (GC), usually with negative or confliciting results (6,22). The commonly recommended initial dose of GC has been 30-40 mg prednisolone per day. Prolonged and continuous corticosteroid therapy should not be used in TA. Instead careful steroid withdrawal should be attempted from time to time with clinical and laboratory monitoring for signs of disease activity. Weekly low-dose methotrexate is now recognized as an effective means of inducing remission and minimizing GC therapy and toxicity in most TA patients (23, 24). The GC-methotrexate combination approach was fairly successful in our patients, but many required surgery for stenotic lesions due to long-standing untreated or unrecognized disease prior to coming to our tertiary center.

Conclusions

We conclude that TA may be an autoimmune disorder targeting an unknown antigen, in which both T cell and humoral aberrations exist. There may be a direct role for cytotoxic CD8 positive T cells in disease pathogenesis, whereas the raised autoantibody and immunoglobulin levels may merely be due to polyclonal hyprgammaglobulinemia consequent to B cell hyperreactivity. The role of antiphospholipid and anti-β2GPI needs further study. A tubercular etiology is unlikely.

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