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Immunological status of patients of Eales' disease

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To test the hypothesis that altered immune reactivity to an extraneous agent might lead to primary retinal perivasculitis, a study was undertaken to determine the serum immunoglobulin levels, T lymphocyte subsets, antibody responses to BCG and 'S' antigen, and lymphoproliferative response to mitogens. No difference was observed in these parameters between patients and controls. Both Mantoux positive and negative conditions existed in patients with Eales' disease. Mantoux positive patients showed a higher level of lymphoproliferative response *in vitro* to PPD than Mantoux positive controls, indicating the presence of two populations among Eales' patients.

Eales' disease is a primary retinal perivasculitis that mainly affects young men between the age of 15 and 35 yr[1-3]. It is now accepted that Eales' disease is a distinct clinical entity. Eales' disease has been reported from many parts of the world, and the condition is more common in Asian countries[4]. Etiology of this disease is still unknown.

It has been suggested by Ashton[5] that Eales' disease is probably the result of localized, chronic low grade inflammatory reaction. Several reports[6-9] show that a number of chronic infectious diseases are associated with Eales' disease, though there is no indication that this clinical condition is due to direct infection of the retina. In recent years, it has been well documented that activated T cells, activated macrophages or locally deposited immune complex, induced localised restricted vascular proliferation[10-13]. In the light of these findings we have proposed that altered immune response of type III (mediated by immune complex) and/or type IV (delayed type hypersensitivity) to extraneous infectious agents lead to the development of localized retinal periphlebitis. We present here the results of our study on lymphocyte subpopulations, general immune status and immune response to PPD and retinal S-antigen in 100 male patients with Eales' disease and 25 controls matched for age and sex.

Material & Methods

Subjects : Patients with Eales' disease were

diagnosed at Aravind Eye Hospital, Madurai. Patients with primary periphlebitis in both eyes or a single eye, both eyes with grade 2 or 3 (moderate or advanced lesions) or one eye with grade 2 and the other eye in grade 3 were chosen for studies. Patients with active tuberculosis, past or present and those with uveal inflammation as well as those with diabetes were excluded. Blood samples were collected from Eales' patients and healthy subjects matched for age and sex. Neither the patients nor the controls were treated with steroid or other drugs, at least 2 months prior to the collection of blood samples.

Mantoux test : This was performed by intradermally injecting 10TU/0.1ml of Tuberculin PPD[14] (Span Diagnostics, India) using tuberculin syringe (Luer Lock). Result of this test was read between 48-72 h after injection. The diameter of the induration was measured in millimeters. Diameter of the induration of more than 10 mm was considered as a positive response.

Immunofluorescence : From fresh blood samples, mononuclear cells were isolated using a Ficoll-Conray gradient and indirect immunofluorescence assay was performed as described earlier[15]. To enumerate total T cells, T4 and T8 positive cells, OKT3, OKT4, and OKT8 monoclonal antibodies from Ortho Diagnostics Inc., USA, were used. GAM-FITC was obtained from Coulter Electronics. Mononuclear cells were observed under phase and UV light (Zeiss Fluoval, excitation filter 224G and barrier filter G247) alternately. At least 200 cells were counted and percentage fluorescence positive cells was evaluated.

Radial immunodiffusion : The total immunoglobulin levels (IgG, IgM and IgA) in the patients and healthy control sera were estimated by the method of Mancini[16]. Gel plates were prepared using agarose type IV (Sigma). The final dilution of class specific antigen (Dakopatts, Denmark) used was 1 : 25. Optimal dilution of sera was used. The diameter of the rings was measured using the partigen scale (Hoechest, India). The values were converted to mg immunoglobulins/ml serum using a standard graph.

Lymphoproliferation assay : The method described by Nussenblatt *et al*[17] was followed. Peripheral blood mononuclear cells (2×10^5 in 200 μl) were cultured in RPMI 1640 (Sigma Chemicals, USA), supplemented with penicillin (100 u/ml), streptomycin (100 μg/ml) (Gibco) and 20 per cent heat inactivated human AB serum. Triplicate cultures were prepared in flat bottom microculture plates (Nunc, Denmark) with or without mitogen or antigen and incubated for 3 or 5 days at 37°C and 5 per cent carbon dioxide and 95 per cent air. Sixteen hour before harvesting, 0.5μCi of tritiated thymidine (BARC, Bombay, specific activity of 6.7 Ci/m mole) was added to the cultures. Radioactivity incorporated was counted in LKB Rackbeta 1211, Wallac liquid scintillation counter.

Mitogens and antigens : Mitogens used were PHA-M (Bacto)—80μg/ml, ConA (Sigma) —30μg/ml, and PWM (Sigma) —5μl/ml. Antigens used were BCG (Guindy, India) —5μg/ml, PPD (Ministry of Agriculture, Food and Fisheries, Weybridge, UK) —20μg/ml and S-antigen 80μg/ml (a kind gift from I. Gery, NEI, NIH; USA)

Enzyme linked immunosorbent assay : To detect antibodies of IgM and IgG isotypes to sonicate supernatant of Bacille Calmette-Guerin, indirect ELISA was used following the method of Voller *et al*[18]. Immunolon plates (Dynatech Laboratory, Inc.) were coated with 5μg/ml BCG sonicate supernatant. After blocking with 1 per cent bovine serum

albumin, serum samples were added to ELISA plates followed by horse raddish peroxidase conjugated anti-human IgG or IgM (Dakopatts, Denmark) at appropriate dilutions. O-phenylene diamine (Sigma Chemicals, USA) was used as substrate, and optical density was read at 490 nm using Dynatech micro Reader II.

Statistical methods : Statistical comparisons were done using Student's 't' test.

Results

All the patients were males and 78 per cent fell within the age group of 15 to 35yr (Table I). They hailed from different parts of India and belonged to different castes and religions. Their socio-economic status was also quite varied. Thus, Eales' disease does not seem to affect any particular population or sect. The clinical examination of Eales' patients revealed that they had no other detectable symptoms of infection or auto-immunity. No evidence was found for association of the disease with any particular profession.

The proportions of different white cell populations in peripheral blood of Eales' patients were within the normal range and comparable to that of healthy Indians (Table I).

The general immune status of Eales' patients characterized on the basis of total T lymphocytes, T4 helper cells, T8 suppressor/cytotoxic cells (Table II) as well

Table I. Age range and total and differential WBC counts in patients of Eales' diesease

Subject	No. studied	Age, yr			Total count (cells/cmm) (mean±SD)	% Differential count (mean±SD)		
		15-24	25-34	35-44		Lymphocyte	Polymorph	Eosinophil
Eales' patients	85	24 (28%)	42 (50%)	19 (22%)	9440±1173	28.4±7.1	64.6±7.3	5.9±4.1
Healthy controls	5	0	5	0	9200±406	33.2±2.4	62.8±0.8	5.6±2.7
Normal range	—	—	—	—	8000 to 10000*	25-35	55-65	0-4

All patients and controls studied were males
*Range as in reference 24

Table II. Lymphocyte subpopulation in Eales' patients
(Data are mean ± SE)

Subject	No. studied	T cells %	B cells %	T4+cells	T8+cells	Ratio T4/T8
Eales' patients	25	57.0±2.0	17.5±1.3	39.9±2.1	28.8±1.6	1.4±0.07
Healthy controls	25	62.6±1.6	16.0±0.8	44.1±1.4	27.6±1.1	1.6±0.07

Table III. Serum immunoglobulin levels in Eales' patients

Subject	Total no.	Serum Ig levels mg/ml (mean ± SE)		
		IgG	IgM	IgA
Eales' patients	25	18.4±1.70	1.5±0.13	1.8±0.14
Healthy controls	22	22.9±1.96	1.4±0.15	2.1±0.27
Healthy adults (India)	—	11.3±4.2 (6.3 to 23)*	1.2±0.5 (0.4 to 2.9)	1.8±0.6 (1.0 to 3.7)

*Range mg per ml as in References 25 and 26

as levels of immunoglobulins (Table III) was not different from that of controls. The lymphoproliferative response to mitogens, PHA-M, Con-A and PWM was strikingly similar in both groups (Fig. 1). The lymphoproliferative response and serum antibody levels to S-antigen of Eales' patients were also not significantly different from those of controls (Table IV).

Table IV. Humoral and cellular responses to 'S' antigen in Eales' patients

(Data are mean ± SE)

Subject	LTT to 'S' antigen	Antibody response to 'S' antigen
	SI	OD values at 490 nm
Patients	2.59±0.660 (18)	0.078±0.02 (15)
Healthy controls	2.24±0.896 (14)	0.070±0.02 (15)

Figures in parentheses indicate total number of samples analysed.
SI, stimulation index

Tuberculin skin test carried out in 88 patients and 21 controls showed a high proportion of Eales' patients being negative to Mantoux test, in contrast to the controls. The proliferative response of lymphocytes to PPD, showed a wide variation in the level of proliferation among Eales' patients (Fig. 2). Though the stimulation index (SI) was higher in Eales' patients than in controls, the difference was not statistically significant. The SI of Mantoux positive patients when compared to that of Mantoux positive controls, showed a highly exaggerated proliferation in many patients of Eales' disease. Most of the Mantoux negative patients showed a very much reduced proliferative response to PPD and SI of Mantoux positive Eales' patients was significantly higher than that of the Mantoux positve controls. These data indicate the existence of two populations of Eales' patients *viz.*, Mantoux positive and Mantoux negative.

Serum samples from the same set of patients and controls analysed for the anti-BCG antibodies of IgM and IgG isotypes (Table V) showed little difference between patients and controls. Additional experiments using immunoblot techniques also

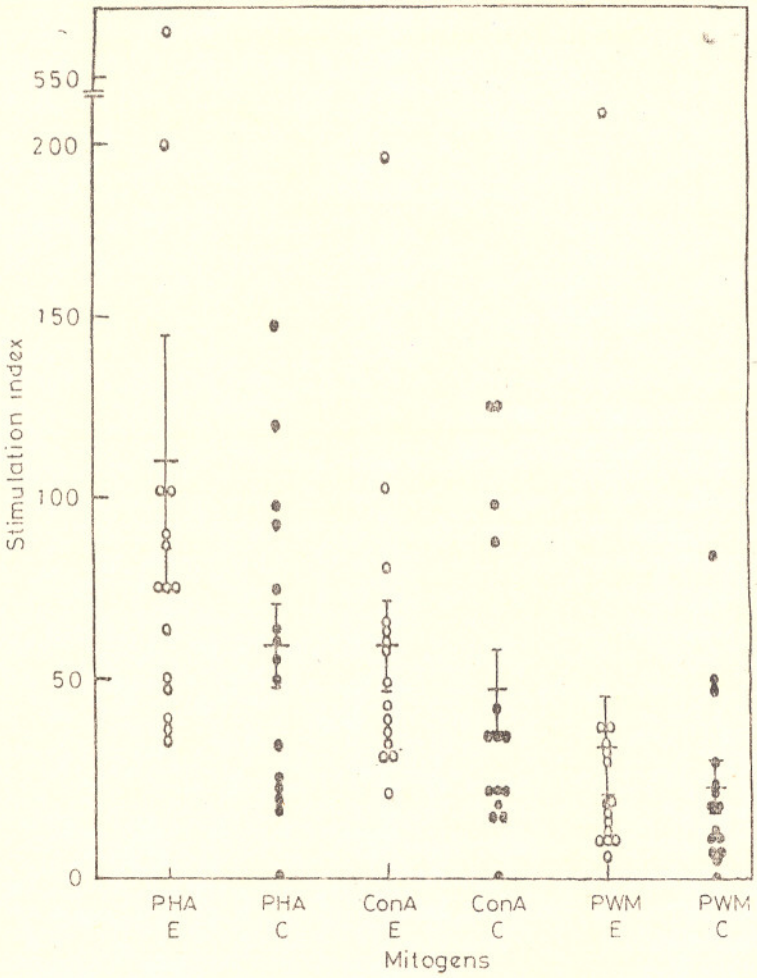

Fig. 1. Scattergram of the stimulation index to mitogens PHA-M, Con A and PWM among controls and Eales' population.
O—Eales' patients; ●—Controls

did not show any qualitative difference in the antibody profile to BCG between Eales' patients and controls (data not shown).

Discussion

The occurrence of periphlebitis in the veins of peripheral retina of Eales' patients was strongly associated with neovascularization and retinal haemorrhage[8]. This condition is even considered to be the earliest lesion in the evolution of Eales' disease and therefore our hypothesis on the etiology of Eales' disease implicates immunological factors. In this context, earlier workers had indicated the importance of allergic

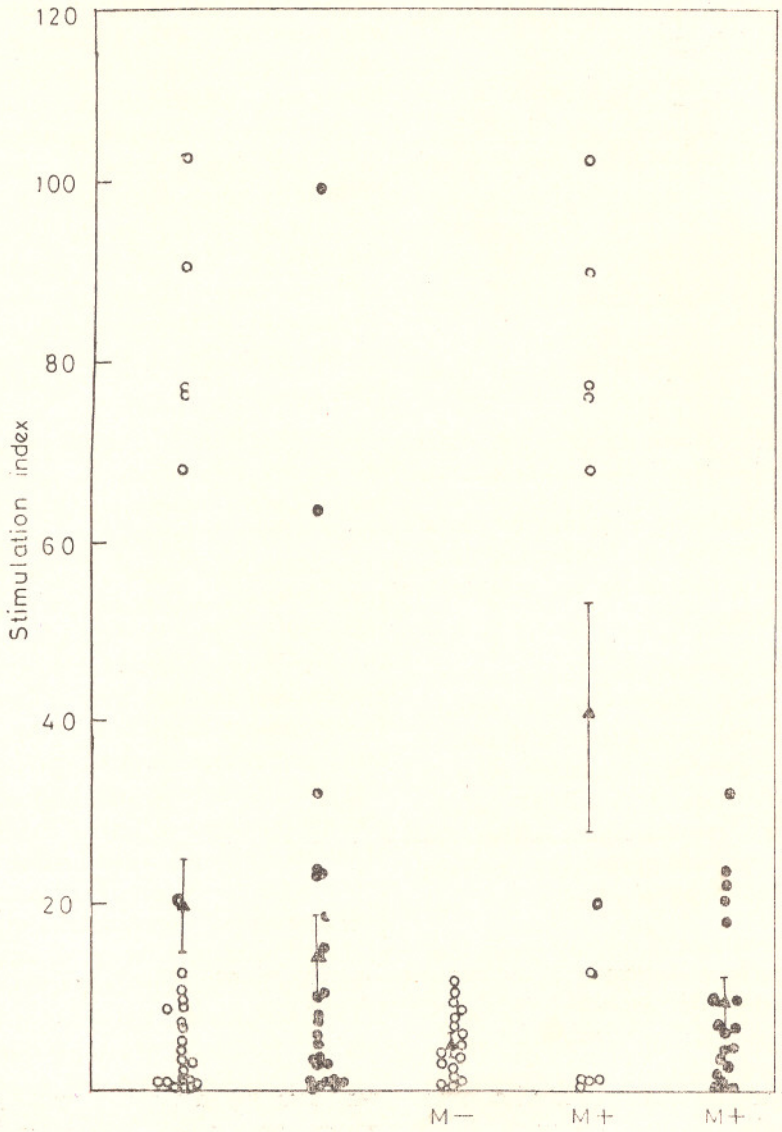

Fig. 2. Scattergram of the stimulation index to purified protein derivative of tuberculin among controls and Eales' population.
O—Eales' patients; ● — Control; M+, Mantoux positive individuals; M—, Mantoux negative individuals

Table V. Antibodies to BCG in Eales' patients

Subject	Total no.	OD value at 490nm (mean± SE) Anti BCG IgG	Anti BCG IgM
Eales' patients	29	0.340±0.059	0.134±0.027
Healthy controls	25	0.280±0.040	0.210±0.040
Eales' Mantoux positive	9	0.385±0.116	0.160±0.080
Eales' Mantoux negative	15	0.280±0.060	0.110±0.023

reactions to tubercular proteins in Eales' disease[19,20]. However, no systematic study has been carried out using an acceptable number of patients.

No defect was observed in Eales' patients with reference to lymphocyte subpopulations in peripheral blood circulation, proliferative response to mitogens and S-antigen; so also in the antibody response to BCG and S-antigen. Earlier reports show either increase or decrease of serum IgG, IgA and IgM levels among Eales' patients[21,22].

However, results of our study imply that a certain specific immunological phenomenon is associated with Eales' disease. The possibility that the patients are sensitized against the retina derived S-antigen can be ruled out since neither antibody response nor lymphocyte proliferative response to this antigen was detected in Eales' patients.

In a recent study Mishra *et al*[20] showed that the majority of Eales' patients (*i.e.*, 7 of 8) were sensitized against tubercular protein (as assessed by the *in vitro* leukocyte migration inhibition test). However, only four of these eight patients were positive for Mantoux test. This finding is of interest, but it should be noted that no control population was included in this study. Results of the present investigation demonstrate that there are two populations among Eales' patients *i.e.*, one postitive and another negative for skin hypersensitive test (Mantoux); and that among most of the Mantoux positive patients, the *in vitro* lymphoproliferative response to PPD was highly enhanced, as compared to that in Mantoux positive controls.

In other studies, the Mantoux sensitivity of healthy individuals in selected parts of India, ranged between 67.4 and 90.35 per cent[23]. Only 20 per cent of our Eales' patients (18 of 88) were Mantoux positive in contrast to 93 per cent positivity in healthy controls (19 of 21). We are aware of the fact that the status of the Mantoux test could be altered by treatment with systemic steroids. In the present study 4 of 16 Mantoux negative Eales' patients and 3 of 9 Mantoux positive patients received steroid treatment which was discontinued at least 2 months prior to Mantoux testing. This finding of a high percentage of Mantoux negativity among Eales' patients is important, as not all patients of Eales' disease have an associated tubercular etiology (except may be those who are Mantoux positive). However, it is advisable that the status of Mantoux test should be reconfirmed, in Mantoux negative Eales' patients by repeating the test at least six months after cessation of chemotherapy.

Eales' patients positive for Mantoux test showed an exaggerated T lymphocyte response *in vitro*. The reasons for this phenomenon are not clear. The role of the mycobacterial antigens in Mantoux positive patients and that of other extraneous factors in

Mantoux negative patients in the altered immune reactivity, calls for further investigations.

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