Immunogenetic studies in retinoblastoma

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SUMMARY In three unrelated families with transmitted retinoblastoma 43 members were tested for HLA-A, B, C, and DR antigens. On 18 patients, 16 unaffected relatives, and 16 controls, mixed lymphocyte cultures were carried out and the response to mitogens, phytohaemagglutinin, concanavalin A, and pokeweed mitogen examined. There were no associations of retinoblastoma with any HLA antigen. A reduction in T cell function is suggested by the results of the mitogen tests.

Retinoblastoma, with an incidence in North-east England of 1 in 16 000, is a malignancy that poses many problems. In some patients it can develop almost at birth and pursue a relentless course despite vigorous and sophisticated treatment, while in other members of the same family the disease, although presumably caused by the same gene, behaves very differently. One member of the first family in the present investigation (II, 12) had a large tumour necessitating enucleation at 6 weeks of age, but a similar tumour in the other eye underwent spontaneous regression. Her sister (II, 14) had one eye completely free of tumour but had a large regressed tumour in the other. A third member (II, 4) had small flat calcified lesions in both eyes, while in the preceding generation the father of these three patients was found at necropsy at the age of 73 to have a dormant, apparently viable, 'retinoma.' Retinoblastoma can present at any age, though usually in infancy; it may affect one or both eyes; it may occur and then spontaneously regress; there may be endophytic, exophytic, or diffuse infiltrating growth; if untreated it is likely to cause death, usually within two years but possibly as late as eleven years from onset, or in a minority of cases an early tumour may regress. There are inherited and non-inherited forms.

To account for the considerable intrafamilial variability that occurs clinically, and the occurrence of occasional spontaneous regression, it has been

suggested that retinoblastoma may have some immunogenetic basis. It is possible that, besides an inherited tendency to maldevelopment of a group of retinal cells, there is also some immunological mechanism which controls tumour proliferation, with some variation in the efficiency of the host immune response to specific tumour antigens. Thus bilateral retinoblastoma would develop where the response is absent, or is too inefficient to control a developing tumour. In some cases of unilateral retinoblastoma the immune response triggered by the initial development would be sufficiently powerful to inhibit tumour development of cells in the other eye. Spontaneous regression would occur when a tumour starts to develop, initiates a highly successful response, is inhibited by it at a critical stage, and then retrogresses. There would thus be an underlying continuum to what appear to be discrete categories of bilateral tumours, unilateral tumours, spontaneous regression, and unaffected carriers. The present study was undertaken to explore this hypothesis further.

Three immunogenetic studies of HLA types in retinoblastoma have been reported. In a group of 122 patients Bertrams *et al.*¹ found a decreased frequency of HLA-B12 and an increased frequency of HLA-Bw35, the latter being significant only among hereditary cases. Segregation analysis of 64 families did not show any significant deviations from expected values. Gallie *et al.*² did not find any significant associations with HLA-A or B antigens in 102 cases. They did, however, observe increased Dw2 and decreased Dw1 frequencies in patients with regressed

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Fig. 1 The three families and the HLA types.

Key to Haplotypes (the gene combination on each chromosome):

Family 1

a: A2, Bw44, DR7	j: A3, B18, DR3
b: A3, B7, DR2	k: Aw30, B7, DRw6
c: Aw23, Bw51, DR5	m: A1, B14, DR blank
d: Aw24, B5, DR1	n: A2, Bw44, DR4
e: A9, B12, Cw1, DR4	o: A2, B8, DR5
f: A9, B27, Cw2, DR5	p: A2, Bw44, DR7
g: A2, B7, DR2	q: A2, B7, Cw2, DR2
h: A3, B7, DR3	r: A2, B27, Cw3, DR4
i: A2, B12, DR1	

Family 2

a: A1, B8, DR7	d: A3, B40, Cw3, DR4
b: A1, B8, DR3	e: A2, B18, DR1
c: A2, B7, DR5	f: A2, B18, DR3

Family 3

a: B14, DR1	c: A11, B27, DR1
b: A2, Bw44, DR2	d: A1, B15, Cw3, DR4

compared with non-regressed tumours. A study³ of some members of the first of the families reported on here showed no evidence of association between

particular HLA haplotypes and retinoblastoma, but suggested decreased responsiveness in mixed lymphocyte cultures of patients by comparison with those in whom spontaneous tumour regression had occurred.

The present study concerns three families in the Newcastle upon Tyne region with transmitted retinoblastoma. The pedigrees are shown in Fig. 1. Their members were typed for HLA-A, B, C, and DR antigens, and one-way mixed lymphocyte cultures (MLC) were set up between members within each family. The mixed lymphocyte reaction is considered to be an in vitro correlate of in vivo graft rejection. In addition cell mediated immunity was investigated in vitro by measuring the lymphocyte transformation in response to stimulation by a standard pool of allogeneic cells and by three mitogens—PHA (phytohaemagglutinin), con A (concanavalin A), and PWM (pokeweed mitogen). PHA and con A are T cell

mitogens, whereas PWM stimulates proliferation of both T and B cells. No patient had received any recent drug therapy likely to affect the results. Controls were healthy unrelated age matched individuals tested in parallel with the families.

Materials and methods

Cell separation. Mononuclear cells were separated from whole blood by density gradient centrifugation, washed, counted, and stored in liquid nitrogen until required. Purified B cells required by HLA-DR typing were obtained following a two-step density gradient centrifugation procedure, in which T cells were separated by E rosetting with sheep red blood cells, and monocytes were removed by an initial incubation with carbonyl iron. B cells were stored at 4°C for three or more hours prior to DR typing.

HLA typing. Samples were tested against a panel of highly purified HLA antisera by a standard two-step microlymphocytotoxicity test, with at least two antisera per antigen.

Mixed lymphocyte cultures. For each test 50 000 responding cells and 50 000 mitomycin-C treated stimulating cells were cultured together in 150 µl culture medium for 120 hours at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. At 97 hours cultures were pulsed with 1 µCi tritiated thymidine; at the end of the culture period cells were harvested, and incorporated thymidine was determined by liquid scintillation counting. For each individual an unstimulated culture of responding cells and culture medium alone, and a standard culture of responding cells and standard pooled stimulating cells, were included. All cultures were performed in triplicate. The results were expressed as median counts per minute (cpm) corrected for background by subtraction of the median cpm of the unstimulated culture. Relative response values were calculated by dividing the corrected response by the corrected response to standard pooled cell stimulation (%RR).

Mitogen test. 50 000 responding cells were cultured with suboptimal, optimal, and supraoptimal concentrations of each of three mitogens, PHA, and con A (90 hours' incubation), and PWM (114 hours' incubation). Incorporation of thymidine was determined as for the MLC test. The results were expressed as

median cpm for each triplicate culture corrected for background by subtraction of the median cpm of unstimulated cultures.

Results

HLA TYPING

The distribution of HLA haplotypes in each family is shown in Fig. 1. For family 1 the typing results were in agreement with those given by Jones.³ There was no evidence of crossovers within the HLA region in any of the families. There was no evidence of association of retinoblastoma with any HLA antigen. Both of the patients with regressed tumours possessed DR2, but so did four of the 10 affected.

MIXED LYMPHOCYTE CULTURES

Table 1 gives the corrected response to standard pooled cells in three groups: patients, unaffected family, and unrelated controls. The mean responses in the patient and family groups were significantly lower than for the control group ($p<0.02$ and $p<0.05$).

Table 2 MLR between individuals with one DR antigen in common

Responders	Stimulators	n	%RR (mean±SEM)
<i>Unrelated</i>			
Controls	Controls*	10	80.3±4.2
Unaffected family	Controls†	16	58.7±5.8
Controls	Unaffected family‡	17	60.4±3.8
Patients	Controls*	16	52.1±4.7
Controls	Patients	12	61.7±7.5
<i>Related</i>			
Unaffected family	Patients	16	40.4±3.5
Patients	Unaffected family	18	38.8±4.2
Unaffected family	Unaffected family	37	39.9±2.8
Patients	Patients	13	33.4±3.7

* $p<0.001$. † $p<0.02$. ‡ $p<0.01$.

Table 3 MLR between individuals with no DR antigen in common

Responders	Stimulators	n	%RR (mean±SEM)
<i>Unrelated</i>			
Controls	Controls	20	87.7±4.1
Unaffected family	Controls	28	88.5±5.4
Controls	Unaffected family	29	85.7±3.7
Patients	Controls	27	79.1±5.2
Controls	Patients	21	77.5±7.4
<i>Related</i>			
Unaffected family	Patients	18	64.1±4.9
Patients	Unaffected family	22	60.4±4.6
Unaffected family	Unaffected family	14	70.4±5.2
Patients	Patients	27	59.7±3.7

No significant differences between groups.

Table 1 Pooled cell stimulation

	n	Mean±SEM	
Controls	29	22 927±1581	$p<0.05$ $p<0.02$
Unaffected family	18	18 313±1366	
Patients	11	16 077± 878	

respectively). The two patients in whom a tumour had regressed lay towards the lower end of the distribution of patient responses. Relative responses in mixed lymphocyte cultures were then grouped according to disparity at the HLA-DR locus; mean relative responses among patients, family members, and controls are given in Table 2 (one DR antigen shared) and Table 3 (no DR antigens shared). There were no significant differences between groups in the

mixed lymphocyte reaction between related individuals. Among unrelated individuals there were no significant differences between groups not sharing any DR antigens. In unrelated groups sharing one DR antigen the mean response in the controls versus controls group was significantly greater than in family versus controls ($p<0.02$), controls versus family ($p<0.01$), and patients versus controls ($p<0.001$).

Fig. 2 Mitogen responses.

MITOGEN TESTS

The mean responses to each mitogen in the patient, unaffected family, and control groups are shown in Fig 2. No significant differences were observed between unaffected family and control groups. However, patients responded less well than controls to mitogen stimulation: they differed significantly in PHA ($p < 0.02$ at $1.3 \mu\text{g/ml}$ and $p < 0.05$ at $3.3 \mu\text{g/ml}$), in optimal PWM stimulation ($p < 0.05$ at $1.7 \mu\text{g/ml}$), and in suboptimal con A stimulation ($p < 0.05$ at $16.7 \mu\text{g/ml}$). Again, the two with regressed tumours were at the lower end of the distribution of responses. The only significant difference between patient and family groups was for PHA at $1.3 \mu\text{g/ml}$ ($p < 0.05$). Multiple linear regression analysis also revealed a significant difference for PHA stimulation between family and patient groups ($p < 0.05$), and between control and patient groups ($p < 0.01$).

Discussion

The possibility of involvement of immunogenetic mechanisms in the development and regression of retinoblastoma has been discussed on several occasions—for example, by Gallie *et al.*⁴ and Roberts and Aherne.⁵ The present results give some support for this.

While HLA typing of the subjects did not reveal any significant non-random segregation within families, and as expected there was no evidence of linkage or association with retinoblastoma, the lymphocyte transformation tests, which measure the afferent arm of the immune response, and the results of the mixed lymphocyte reaction, are more positive.

The three mitogens used here stimulate different but overlapping lymphocyte populations. PHA mainly stimulates proliferation of the majority of T cell subpopulations; con A is also a T cell mitogen, which activates different subpopulations at different doses—helper cells at suboptimal doses, and most T cell populations at optimal dose. PWM stimulates both T cells and B cells. In mixed lymphocyte cultures the response to a standard pool of stimulating cells is a measure of the ability to respond, by T helper cell proliferation, to allogeneic challenge.

The results indicate that in vitro lymphocyte transformation in response to these non-specific mitogens is reduced among retinoblastoma patients compared with controls. Mean responses among unaffected family were intermediate between the patient and control groups. Moreover the reduced MLR to allogeneic cells not only confirms but goes further than the results of the earlier study of family 1,³ for it takes into account the fact that the extent of the reaction reflects the degree of histocompatibility between the individuals tested, and especially at the

HLA D/DR locus. Hence MLC comparisons need to be made within groups of subjects with similar degrees of disparity—that is, having two haplotypes in common, one haplotype, or no haplotype in common. In the present study significant differences occurred in the one haplotype disparate group, where the mean response in patients versus controls, unaffected family versus controls, and controls versus unaffected family was below that in controls versus controls. The responses to the mitogens and allogeneic cells in combination suggest a general depression in cell mediated immunity, and particularly a reduced T cell function in patients.

No studies of general cell mediated immunity in retinoblastoma have been reported, most immunological studies focusing on the reactivity of patients to tumour cell lines and the characterisation of immune complexes,^{4,6-8} and as yet the antigens in such reactions are poorly characterised. However, a reduction in cell mediated immunity has frequently been observed in patients with other malignant disease, such as chronic lymphocytic leukaemia, Hodgkin's disease, and carcinoma of the breast, lung, and colon.⁹⁻¹¹ There are conflicting opinions on whether this degree of depression correlates with disease activity.¹²⁻¹⁴ Initially it was assumed that the reduced cell mediated immunity resulted from an impairment of the cell populations directly involved in the responses being measured. Today, however, there is increasing evidence that these cell populations are functionally intact but are actively inhibited by the action of suppressor cell populations, particularly suppressor T cells and monocytes, for their removal often restores in vitro proliferative capacity to normal levels in most patients.

The reduction in in-vitro lymphocyte proliferation in retinoblastoma patients in the present study might be mediated by active suppressor mechanisms as described in other malignancies.¹⁵⁻¹⁸ However, alternative explanations must also be considered. There might be a reduction in the number of circulating T cells, or an alteration in the ratio of T cell subsets, or there could be a specific defect in the proliferative capacity of the responding T cell populations. There is no evidence of a specific impairment in the ability to recognise cell surface antigens.

In the mitogen and MLC tests the lower level of response in the two with regressed tumours may be partly a function of their older age. However, Berlinger and Good¹⁹ studied cancer free individuals from family aggregates of hereditary colonic carcinoma and found that in individuals with no evidence of precancerous disease the response to allogeneic stimuli was decreased. This deficiency could in some cases be attributed to the suppressive influence of adherent cells (monocytes) on normally responding

lymphocytes. A similar pattern was observed in the present retinoblastoma families. Unaffected family members showed a significantly reduced response to allogeneic cells but did not differ in mitogen responsiveness. This group was heterogeneous, since it included both those without retinoblastoma and the silent transmitters, and it would be worth inquiring whether the reduced cell mediated immunity is restricted to the latter.

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