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Immunological Paradox in Testicular Tumours: the Presence of a Large Number of Activated T-cells Despite the Complete Absence of MHC Antigens

A.M.E. Nouri, R.F. Hussain, R.T.D. Oliver, A.M. Handy, I. Bartkova and J.G. Bodmer

Tissue sections from 22 seminoma (Se) and 10 teratoma (Te) patients were investigated for correlation between the presence of tumour infiltrating T-lymphocytes (TIL) and the expression of major histocompatibility complex (MHC) antigens using an immunoperoxidase staining technique. Complete absence of both class I and II antigens was observed in all Te and 20 out of 22 Se. The two positive Se showed only weak expression on 2% of tumour cells. Despite the absence of human leucocyte antigens (HLA) there were a large number of TIL scattered throughout the tissues in the case of Se with no predominance of CD4 or CD8 subpopulations in either group. Tγ positive cells were less than 5% of total CD3 positive cells in both Se and Te. The majority of the TIL were found to express activation markers, i.e. HLA class II antigens. Culture of tumour cell suspension with IL-2 produced passageable IL-2-dependent T cells from 10 out of 15 tumours. Studies with testis cell lines showed the complete absence of class I antigens in 2 out of 5 cases and the inability of interferon γ (IFN γ) to induce expression. IFN γ also failed to induce class II antigens in three out of five of these lines. The immunological paradox of the presence of a large number of activated T-cells in testicular tumours despite the complete absence of MHC antigens remains unexplained and needs further investigation. Possible hypotheses are reviewed.
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INTRODUCTION

MAJOR HISTOCOMPATIBILITY complex (MHC) antigens are cell surface glycoproteins which play a central role in the initiation of immunological responses. Apart from sperm [1] and brain [2], class I antigens are expressed on all the human nucleated cells whereas class II are only expressed on B-cells, antigen presenting cells like macrophages and activated T-cells [3].

Zinkernagel and Doherty [4] demonstrated that these antigens act as restriction elements for presentation of non-self antigens to T-cells. The importance of class I and II antigens for regulating resistance to viral infection [5] as well as graft and tumour rejection in experimental animal models [6, 7] has since been well documented.

Loss or diminished class I antigens from tumours have been reported by some [8, 9] but not by others [10] to correlate with increased invasive malignancy. This has led to the suggestion that these abnormalities may be a factor in escape from immune surveillance. The situation with class II antigens is more complicated. Some authors reported failure of tumour cells to express class II antigens following interferon gamma stimulation [11, 12]. Alexander *et al.* [13] showed over-expressed, non-functioning class II antigens in melanoma while Rubin *et al.* [14] reported normal class II expression in these patients which was a good predictor for response to cytokine therapy.

If the MHC antigen abnormality is an important factor for tumour escape, the absence of these antigens from spermatogonia and brain cells is surprising, as the tumours in these organs are no more frequent than in any other organ. Further confusion to the immune surveillance hypothesis comes from the observation that good prognosis correlates with the degree of lymphoid infiltrate in seminoma [15], where there is complete absence of MHC antigens [16] and spontaneous regression of the primary tumour is most frequent [17].

This study was aimed to confirm the reported lack of expression of MHC antigens in germ cell tumours and cell lines developed from these tumours; to induce these antigens by cytokines on the cell lines and investigate the state of activation and phenotypes of infiltrating T-cells.

MATERIALS AND METHODS

Interferons, monoclonal antibodies and plasmids

Interferon (IFN) α and γ was obtained from Wellcome and Biogen, respectively. Monoclonal antibodies (Mabs) were W6/32 [18], which detects all beta 2m-associated human leucocyte antigen (HLA)-A,B,C antigens, HC10 [19], which detects class I free heavy chains and HB55 (ATCC [20]), which detects HLA-DR antigens. CD3, CD4, CD8 and Tγ were obtained from Beeton Dickinson (Diagnostic Systems).

Development of cell lines

Established cell lines were developed as described previously [21]. Cell lines Ha (teratoma) and Lan (seminoma), were estab-

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lished and kept in culture as described by Nouri *et al.* [12]. For Tera I, Tera II and Ep2102 cell lines see Iles *et al.* [22].

Immunocytochemistry

Surgically removed tumours from 22 seminomas (Se) and 10 teratomas (Te) were snap frozen and kept in liquid nitrogen until use. Tissue sections (7 μm) were cut, air dried, acetone fixed and after hydration were used for immunoperoxidase staining as previously described by Nouri *et al.* [21].

Binding assay

Tumour cells (1×10^4/well) were treated with IFN α (1 000 μ/ml) and γ (100 μ/ml) for 48 h (concentrations which were previously found to be optimum for maximum class I and II antigen induction [12]) in flat-bottomed microtitre plates. Appropriate concentrations of specific Mabs (50 μl/well, in triplicate) containing 0.02% sodium azide were added and incubated for 45 min at room temperature.

After three washes, 50 μl of diluted [in RPMI plus 10% fetal calf serum (FCS) and 0.02% azide] iodinated rabbit-anti-mouse antibody (50 000 cpm/well, Amersham) were added and incubation continued for a further 45 min. Following three washes, the cells were lysed with 100 μl/well of 2% (v/v) Triton X100 in water and the degree of radioactivity in the supernatants was measured using a gamma counter.

RESULTS

Expression of class I *antigens on testicular seminiferous tubules*

Tissue sections from nine testes tumour biopsy specimens showing "normal" residual tubule areas with microscopically normal morphology were used for assessing the expression of free heavy and light chains of class I antigens. Although the intensity of staining differed from individual to individual, all three Mabs W6/32, HC10 and BBM.1 showed positive staining with the stromal cells. HC10 was the only Mab which showed positive staining with germ cells within seminiferous tubules, an example of which is shown in Fig. 1a and b.

Expression of MHC antigens on testis tumour sections

In two of the 22 Se tested, Mabs W6/32, HC10 and BBM.1 showed a small number of weakly stained tumour cells (one to two cells/field) in both cases (Table 1, Fig. 2a and b). In the case of the class II antigens, two out of 17 showed a small number (one to two cells/field) of positively stained tumours. The pattern of expression of these antigens on 10 Te tumours was very similar to Se, indicating that within the sensitivity limit of the peroxidase staining technique Se and Te tumours are negative for both class I and class II antigens.

Expression of T-cell activation markers on infiltrating T-cells

Tumour infiltrating T-lymphocytes (TIL) were found to be present in all the 22 Se and 10 Te tumours to varying degrees with Se showing a greater infiltration number than Te (Fig. 3a and b). *In vitro* interleukin-2 (Il-2) stimulation of T-cells isolated from 10 of 15 of these tumours was successfully expanded for different length of time (Table 2). Unlike Te in which they appeared focally, T-cells tended to be scattered evenly throughout the tissue in Se (Fig. 3a and b).

In addition, the analysis of tissues for T-cell subtypes showed that there was no predominant infiltration of either CD4 or CD8 positive cell populations in any of the tumours in either group. Similarly, the percentage of T γ positive population was also found to be within the normal range (2–5% of total T-cells, similar to that seen in peripheral blood cells). When sequential staining for CD3 and class II antigens (as detected with HB55 Mab) was carried out it was found that in both groups the majority of infiltrating T-cells were class II positive (Table 3) indicating the activated nature of these T cells.

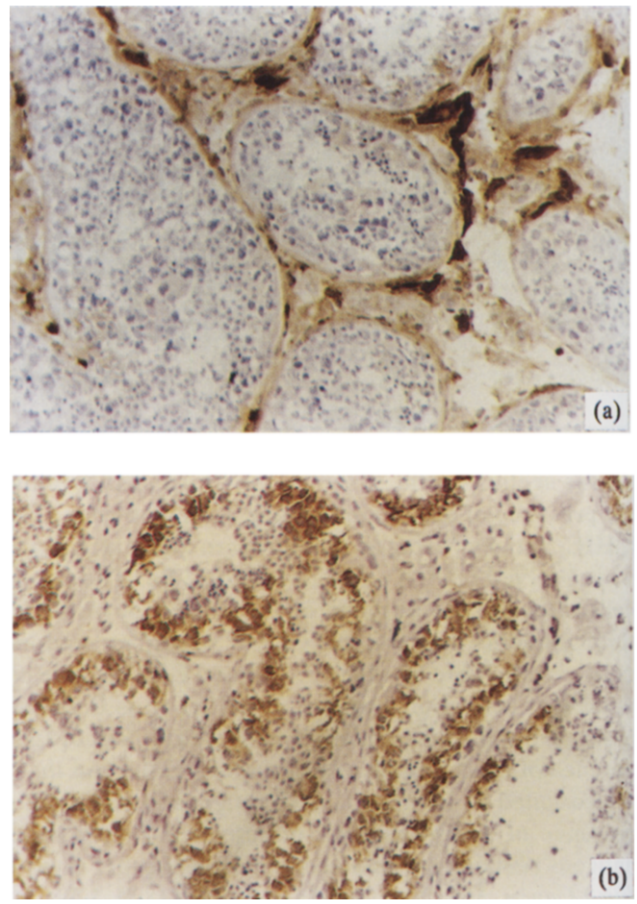

Fig. 1. (a) and (b) Staining with W6/32 and HC10 Mabs on morphologically normal testis tumour sections.

Induction of MHC antigens by cell lines in response to IFN γ

The inducibility of MHC antigens by tumour cell lines in response to IFN γ was investigated using a radiolabelled binding technique and the results are expressed in cpm. Values below

Table 1. *Expression of MHC antigens on seminoma and teratoma tissue sections*

Antibody	Seminoma	Teratoma
W6/32	2/22* (9%)	0/10 (0%)
HC10	1/22* (4%)	0/8 (0%)
BBM.1	1/22* (4%)	0/10 (0%)
HB55	2/17* (11%)	0/7 (0%)

Results in parentheses are expressed in per cent positive cases. * Indicates cases where 1–2% of total cells are positive.

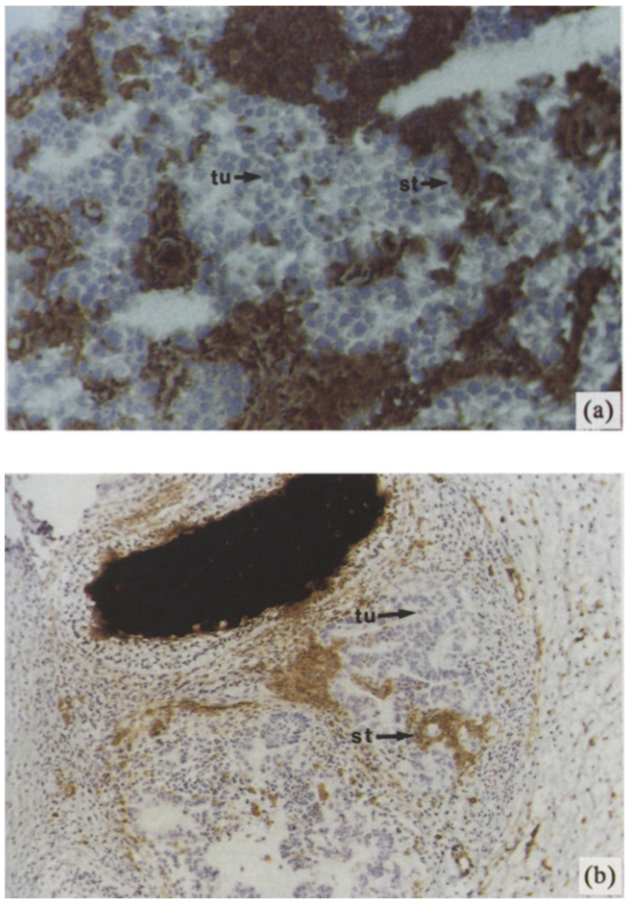

Fig. 2. Staining of seminoma (a) and teratoma (b) tissue sections with W6/32 Mab. Arrows show positive stroma (st) and negative tumour cells (tu).

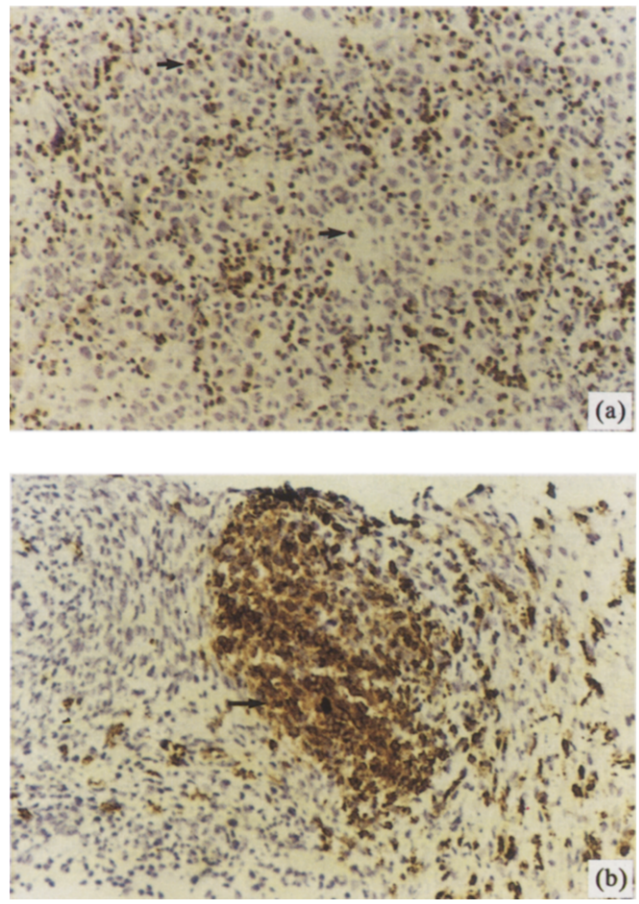

Fig. 3. Staining of seminoma (a) and teratoma (b) tissue sections with anti-CD3 Mab. Arrows show CD3-positive infiltrating T-cells.

150 cpm indicate background counts and therefore show negative binding.

As can be seen from Table 4, both Tera I and Ha cell lines showed negative staining with W6/32 and remained negative after IFN γ stimulation whereas under the same conditions IFNγ induced class II antigens in both cases. In contrast, Tera II and EP2102 which showed very low levels of class I antigens were upregulated after IFN γ stimulation, whilst under the same conditions their class II antigens were not induced. These results are indicative of the variable nature of class I or II antigen deficiency in testis cell lines and inability of IFN γ to correct the deficit.

DISCUSSION

There are five conclusions drawn from the findings of this investigation: (a) neither seminomas nor teratomas express MHC antigens. (b) There are infiltrating T-cells in both tumour types, most of which express class II antigens indicating that they are activated. (c) The primary stem cells within the seminiferous tubules showed positive staining with HC10 detecting free heavy chain of class I antigens though none of the seminomas or teratomas showed positivity with this antibody (d) Two out of five lines showed absence of class I antigens which was not corrected by IFN α or γ. (e) In 3 out of 5 cell lines, IFN γ failed to induce class II antigens.

These observations support the notion that spermatogonia as well as testicular germ cell tumours and some cell lines derived from them have reduced or absent MHC class I antigens,

Table 2. Length of incubation time for TIL expansion in culture with IL-2

Name	Case	Length of culture (in days)
AG	Te	No TIL
CC	Te	35
CCh	Se	16
DM	Se	8
EW	Se	No TIL
GF	Se	33
JB	Se	Contamination
JP	Se	32
JW	Se	No TIL
MC	Se	16
NT	Se	13
RL	Se	35
SH	Se	No TIL
TH	Te	35
TM	Se	11

Cells were fed with IL-2 (100 U/ml) every 2–3 days.

Table 3. Expression of T-cell markers

Marker	Seminoma	Teratoma
CD3	22/22 (100%)	10/10 (100%)
CD3 + HB55	22/22 (100%)	10/10 (100%)
CD4	17/17 (100%)	8/8 (100%)
CD8	17/17 (100%)	8/8 (100%)

Results are expressed in per cent positive cells.

Table 4. Induction of MHC antigens in testis cell lines in response to IFN

	Class I		Class II	
	Control	IFN γ	Control	IFN γ
Tera I	63 ± 12	65 ± 30‡	40 ± 9	677 ± 90*
Tera II	240 ± 49	1560 ± 139*	59 ± 34	93 ± 26‡
EP2102	660 ± 108	1222 ± 149*	92 ± 72	130 ± 32‡
Lan	799 ± 76	680 ± 32‡	48 ± 8	89 ± 22‡
Ha	84 ± 14	60 ± 37‡	54 ± 12	1005 ± 20*

Results are expressed as mean ± S.D. in three replicates (cpm). *, † and ‡ indicate P values of 0.001, 0.01 and not significant, respectively.

confirming previous reports of Bell *et al.* [16] and Klein *et al.* [23]. Our observations that carcinoma *in situ* cells in tubules adjacent to germ cell tumours express class I free heavy chain is interesting and confirm those of Stam *et al.* [24] who demonstrated these molecules on spermatogonia in morphologically normal seminiferous tubules. As yet this is unexplained, but it does suggest that there is a need for a more detailed study using molecular biology approach employing MHC-specific mRNA probes.

There have been two reports correlating the degree of HLA antigen loss from adult solid tumours with lack of response to Il-2 [14, 21]. The most significant unexplained observation reported in this paper is the finding that despite the total lack of HLA class I antigens on germ cells, the TIL express activation marker suggesting that they may be involved in active immune response. This finding is consistent with the long standing observation that germ cell tumours show the highest correlation between the degree of lymphocyte infiltration and survival [25] as well as demonstrating the highest incidence of spontaneous regression of the primary tumour [26]. The low level of T-cell receptor gamma on the T-cells would suggest that it is T-cell receptor α β which is engaged and needs further studies using specific antibodies against polymorphic T-cell receptors.

There are two possible hypotheses to explain the inconsistency of the presence of activated T-cells in testicular germ cell tumours despite the complete absence of class I antigens. Either the T-cells are activated using a non-classical MHC class I antigens as restriction element or they are simply non-specifically activated with the cytokines generated from tumour apoptosis [27]. In favour of the first hypothesis is the fact that the nature of the infiltrate differs little from that seen in rejecting transplants. Of particular relevance to the issue of germ cell tumours, is the recent evidence that trophoblasts rather than totally lacking MHC class I antigen as previously thought, expresses a non-classical HLA-G [28] antigen.

Further complexicity regarding the non-specific infiltration of T-cells has been described by Grossman [29] demonstrating the role of sex hormones in regulating the thymus involution and the levels of circulating lymphocytes. There is now clear cut evidence from experimental animal studies that castration of adult animals leads to thymic regeneration and increasing levels of circulating lymphocytes. Possible evidence that such an effect can occur in man comes from two studies currently in progress in our department. The first observation comes from a study demonstrated that surgical castration of patients with prostate cancer is associated with an increase in circulating lymphocytes (Joseph and Oliver, in preparation). In the second study testis cancer patients developing thymic enlargement after chemotherapy have been found to show testicular atrophy leading to the lowering of testosterone and elevated follicular stimulating hormone (Sperandio and Oliver, in preparation).

In conclusion, this paper provides the first unequivocal evidence of activated T-lymphocytes infiltrating germ cell tumours. Though the mechanism of T-cell activation is not clear, there might be two possible explanations. First, the involvement of non-classical MHC antigens acting as restriction element and secondly, the presence of cytokines in the vicinity of tumour following tumour apoptosis.

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Intravesical Mitoxantrone in Superficial Bladder Tumours (Ta–T1)

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36 patients with histologically proven grade G1–G2, Ta–T1 transitional cell carcinoma of the bladder were introduced, after transurethral resection (TUR), into a study of intravesical chemoprophylaxis with mitoxantrone (20 mg diluted in 50 ml). After a mean follow-up of 23 months, 16 (50%) patients showed a superficial recurrence with a mean recurrence rate of 0.56 per year. In 19 patients with recurring tumours the mean recurrence rate decreased from 1.65 to 0.58 per year. 9 patients (25.7%) suffered from a chemical cystitis that in 2 cases (5.7%) required treatment interruption.
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INTRODUCTION

ALTHOUGH INTRAVESICAL treatment with BCG doxorubicin, epirubicin, ethoglucide, thiotepa or mitomycin C has been shown to reduce recurrence rate of superficial (Ta, T1) bladder tumours, a substantial number of patients will continue to show recurrent lesions and some may undergo tumour progression and death. As discussed in previous papers [1, 2] there is an urgent need for more information on new modalities of treatment and to test new anticancer drugs for their potential intravesical use.

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Mitoxantrone is an anthraquinone derivative, related to daunorubicin, doxorubicin and epirubicin. It differs from these in that it has a more intense and broader antitumour activity *in vitro*. It acts on neoplastic cells at phase G0, being able to interact not only with DNA but also with mRNA [3, 4].

A recent phase I study of intravesical chemotherapy suggested some activity of mitoxantrone on vesical transitional cell tumours and indicated that 10 mg (diluted in 30 ml of saline solution) was the highest tolerable dose [5]. Since the antitumour activity of mitoxantrone was proven to be dose-related *in vitro*, we initiated a pilot study to test the tolerability and the efficacy of mitoxantrone at a higher dose than previously reported and adopting a different retention time.

MATERIALS AND METHODS

36 patients with histologically proven grade G1–G2, Ta–T1 transitional cell carcinoma of the bladder, removed by trans-