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An Immunohistopathological Characterisation of Mixed Non-Seminomatous Germ Cell Tumors

E. Gemmell, G. M. Strutton, G. J. Seymour, M. D. Walsh, M. F. Lavin and R. A. Gardiner

Department of Surgery, University of Queensland, Royal Brisbane Hospital, Herston, Australia

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Summary. Immunohistological techniques were used to characterise inflammatory cell infiltrates in mixed germ cell tumours. The distribution of these infiltrates was much more variable than in pure seminomas but could not be related accurately to any particular tumour type. There were approximately equal numbers of B and T cells in these areas and helper/inducer T cells were more common than suppressor/cytotoxic lymphocytes. Within these areas of inflammatory cells, the subtype composition was similar to that seen in pure seminomas.

Key words: Immunohistology – Non-seminomatous germ cell tumors

Introduction

Until recently it was difficult, if not impossible, to distinguish between differing populations and subpopulations of immunocompetent cells on morphological grounds alone, However, since the development of immunohistological techniques, identification of these cells together with establishment of their in-situ micro-environments have become feasible. The development of monoclonal antibodies which recognise functional subpopulations of T-cells has been a major advance and these antibodies have been employed to examine a wide variety of conditions [8, 12]. Secondly, appreciation of heterogeneity within the macrophage population has contributed greatly as it is now realised that the non-lymphoid cells found in the T-cell areas of lymphoid organs are distinct from classical histiocytes and from inflammatory macrophages. These interdigitating (ID) reticulum cells express HLA-DR antigens strongly, but are not phagocytic, containing only small amounts of lysosymal enzymes such as acid phosphatase (ACP). They do, however, have readily identifiable surface membrane adenosine triphosphatase (ATPase). These cells are thought to play a major role in presenting antigen to T-cells. Finally, a method has been developed enabling identification of two populations of cells in the same section [12]. These immunofluorescence and immunoenzyme techniques have also been interfaced with cytochemical reactions, so that, in combination, they permit recognition of cellular inter-relationships [7].

In primary seminomas of the testis, lymphocytic infiltration is a common finding and the relatively favourable prognosis of patients with this tumour has been attributed, at least in part, to this feature [3, 9, 16]. In contrast, non-seminomatous germ cell tumours (NSGCT) constitute a much more heterogenous subgroup with considerable variation in lymphocyte numbers [2, 3, 10]. In a recent study, the inflammatory cell infiltrate in classical seminomas was examined by immunohistological techniques. T cells outnumbered B cells in all sections studied. The helper/inducer $CD4^+$ subgroup of T lymphocytes predominated, but, within the $CD8^+$ population, suppressor cells were more common than cytotoxic cells. Phagocytic macrophages were twice as common as antigen presenting macrophages, which were nevertheless found in association with large numbers of T lymphocytes [15]. The significance of these findings remains speculative but does serve as a basis for further functional analysis.

The aim of this study was to examine NSGCT by similar techniques to identify the cells of immunological lineage in these tumours. Again, the emerging immunohistological picture will serve as a reference for subsequent studies.

Materials and Methods

Patients

Fresh tissue was obtained at the time of operation from six patients who had radical orchidectomies for nonseminomatous germ cell

Address for reprints: Dr. R. A. Gardiner, Department of Surgery, University of Queensland, Clinical Sciences Building, Royal Brisbane Hospital, Herston, 4029, Australia

Table 1

Patient	Histopathology	HCG	AFP	Total LD	LD 1 & 2	Stage
A	Mature & immature teratoma	n	+	n	n	II
B	Mature teratoma Embryonal ca.	+	+	+	n	III
C	Mature & immature teratoma Embryonal ca. Choriocarcinoma	+	+	+	+	III
D	Mature & immature Embryonal ca. Yolk sac tumour	+	+	n	n	I
E	Embryonal ca. Choriocarcinoma	+	+	n	n	II
F	Yolk sac tumour Embryonal ca.	+	+	n	n	III

(Histological classification based on Mostofi, reference 10)

Table 2. Monoclonal antibodies

Cell type	Cluster designation (CD)	Antibody	Source
B cells		Leu14	(Becton-Dickinson)
Pan T	CD3	Leu4 OKT3	(Becton-Dickinson) (Orthomune)
Helper/inducer T cells	CD4	Leu3 OKT4	(Becton-Dickinson) (Orthomune)
Suppressor/cytotoxic T cells	CD8	Leu2 OKT8	(Becton-Dickinson) (Orthomune)
Suppressor T cells		Leu15	(Becton-Dickinson)
Natural killer cells		Leu7 & 11b	(Becton-Dickinson)
Langerhans cells	CD1	OKT6	(Orthomune)
MHC class I antigens		HLA-ABC	(Serolab)
MHC class II antigens HLA-DQ HLA-DR		Leu10 HLA-DR	(Becton-Dickinson) (Becton-Dickinson)

tumours. Macroscopically identical tumour tissue was selected for immunohistology and routine histopathology. Biopsies of an overtly normal testis were collected immediately prior to surgical correction of a primary, idiopathic hydrocoele. Identical specimens were sent for both routine histopathology and immunohistology.

Component tumour types, tumour marker status and clinical stages are listed in Table 1.

Selection and Preparation of Tissues

The tissue preparations and staining were carried out as described previously by Poulter et al. [11]. Tumour was sectioned and a macro scopically representative area was selected for immunohistology. Adjacent tissue, similar to that chosen for immunohistology, was fixed in formalin, paraffin embedded, sectioned then stained with haematoxylin and eosin. The tumour tissue for immunohistology was quenched in liquid nitrogen after embedding in OCT then stored at −70 °C prior to cutting into 6 micron sections with a Damon/I.E.C. cryostat. Sections were dried for 2 h at room temperature, then fixed in equal parts chloroform/acetone for 5 min, individually wrapped in plastic film and stored at −20 °C.

Monoclonal Antibodies

A panel of monoclonal antibodies (MoAbs) with well defined specificities was used. These are listed in Table 2.

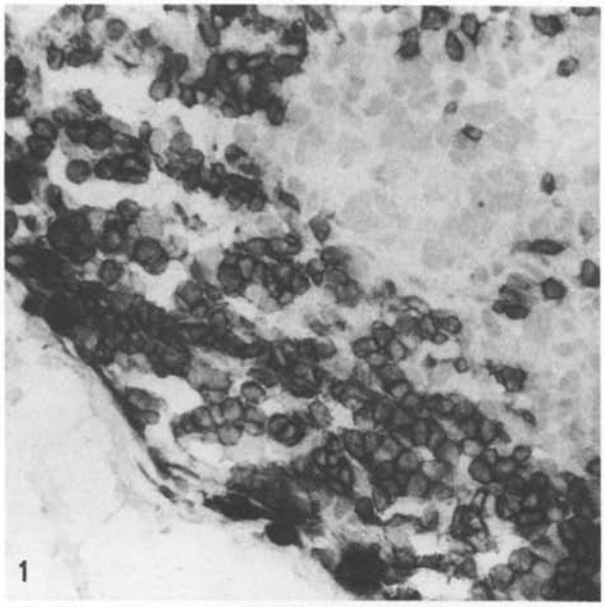
Immunoperoxidase Techniques

The above MoAbs, with the exception of anti Leu15, were used in an avidin biotin immunoperoxidase method. Frozen sections were first incubated with the MoAb of choice at a dilution of 1/10 followed by a 1/20 dilution of affinity purified biotinylated sheep anti mouse Ig (Amersham) and finally with a 1/50 dilution of streptavidin peroxidase (Amersham). Each incubation was carried out for 30

Table 3

Patient	Mean T & B cell no./HPF	T/B Ratio	CD4/CD8 ratio
A	320	0.82:1	1.37:1
B	300	0.94:1	1.78:1
C	300	1.11:1	1.65:1
D	150	3.68:1	1.76:1
E	90	1.93:1	1.49:1
F	250	1.13:1	1.32:1
Mean		1.60:1	1.56:1
Standard error		0.44	0.08
Standard deviation		1.09	0.20

(× 1 High Power Field = 0.0987 mm^2)



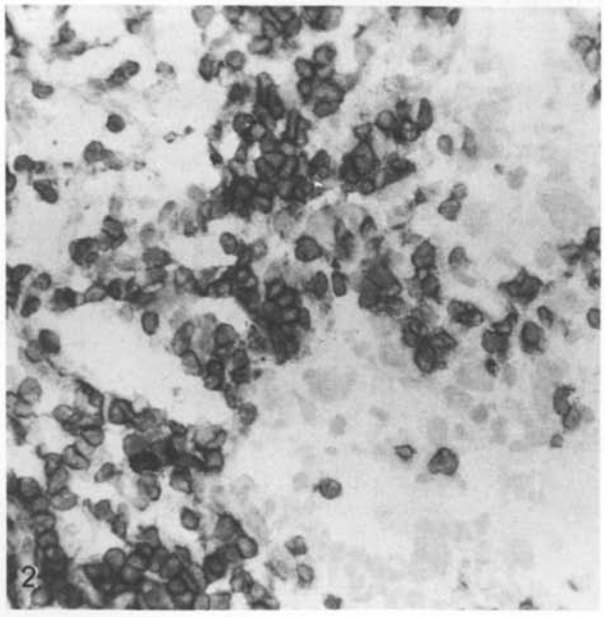


Fig. 1. Leu4 positive cells (pan T). Immunoperoxidase x320

Fig. 2. Leu14 positive cells (B cells). Immunoperoxidase x320

min at room temperature followed by washing for 10 min in phosphate buffered saline pH 7.2 (PBS). Staining was achieved by immersing sections in 0.05% 3,3'-diaminobenzadine (Sigma) in TRIS-HCL buffer, pH 7.6, containing 0.001% hydrogen peroxide [5]. Nuclei were counterstained with Mayer's haematoxylin. Optimal dilutions of each layer were predetermined using frozen sections of human tonsils as positive controls [14]. Negative controls were prepared by omitting the primary MoAb layer or using an irrelevant monoclonal antibody FNBA4 [1], all other steps in the method having been described above.

Immunofluorescence

Fluorescence conjugated OKT8 and phycoerythrin conjugated anti-Leu15 were employed in a double staining immunofluorescence technique to distinguish the suppressor subpopulation within the $CD8^+$ population. Sections were incubated with the former antibody at a dilution of 1/10 for 1 h at room temperature, washed in PBS and then incubated with the latter antibody under the same conditions. After a final washing with PBS, sections were mounted in PBS:glycerol (30:70) and viewed using a Zeiss Epifluorescent microscope with narrow band FITC (green) and TRITC (red) filters.

Histochemistry

A simultaneous azo dye coupling technique using naphthol AS-BI phosphate as substrate and hexazotised pararosaniline as the dye, was used to stain for acid phosphatase (ACP) containing macrophages. A lead precipitate method for magnesium activated adenosine triphosphatase (ATPase) was employed for the demonstration of ATPase positive macrophages [11].

Cell Counting Procedures

For each of the MoAbs, Leu14, Leu4, Leu3, Leu2, Leu7, Leu11b, I1-2 receptor and OKT6, 10 fields (area of 1 field = 0.0987 mm^2) in areas infiltrated heavily with lymphocytes were counted. The ratios between T helper/inducer and T suppressor/cytotoxic lymphocytes and between T and B cells were calculated.

Results

A variation in inflammatory cell numbers was noted throughout the tumours examined; there was a scarcity in some areas and a preponderance surrounding tumour lobules in others. This variation could not be related to NSGCT subtypes accurately since malignant cells constituted faint background in the study sections and frozen section diagnosis of immediately adjacent sections was not absolutely reliable both in terms of sampling and morphological integrity. The areas of maximum inflammatory infiltrate were counted and the results are presented in Table 3.

With respect to the T cell : B ratio, only one specimen showed a ratio over 3:1 (Table 3) while in all other specimens almost equal numbers of T and B cells were found (Figs. 1, 2). Within the T cell population, both $CD4^+$ and $CD8^+$ T cells were studied in all specimens with only a minor variation in CD4:CD8 ratio (Table 3).

Double staining immunofluorescence demonstrated that most $CD8^+$ cells in all the testes were also $Leu15^+$ with

the exception of the first tumour (A in Table 1) in which most $CD8^+$ cells were not $Leu15^+$. In the tumours less than 3% of the lymphocyte population were $Leu7^+$, the Leu11b cells forming a smaller proportion. The only $DC1^+$ cells identified in this study were in sections from testes tumour patients (A, B, E). Occasional plasma cells were seen in tumour sections but not in other sections examined.

Class I and Class II antigen expression was identified on endothelial and mononuclear cells in all specimens. Tumour cells never expressed DQ antigens, however, some tumour epithelial cells were seen to express ABC and DR antigens. Dendritic cells with a phenotype HLA-ABC^-, HLA-DR^+, HLA-DQ^-, $CD1^-$ ($T6^-$) were present in only one of the tumours (C in Table 1).

Tumour cells and mononuclear cells expressed transferrin receptor (TR^+). Very few $ATPase^+$ macrophages were found in the inflammatory infiltrates in the tumour sections (none being found in 3 of the 7 specimens, approximately 1:200 lymphocytes in a further 2 tumours and approximately 1:15–40 T cells in the last 2 tumours), however in most of these malignancies ACP^+ macrophages were not uncommon.

Discussion

In contrast to seminomas, lymphocytic aggregates seldom receive particular mention in histopathological resports of non-seminomatous germ cell tumours of the testis. In this study the numbers of lymphocytes in the areas of highest concentration in most cases exceeded those counted surrounding tumour lobules in classical seminomas. In pure seminomas previously studied, lymphocytic aggregates were evident in all parts of the slides in similar concentrations surrounding islands of tumour [15]. Areas of necrosis were more commonly seen in non-seminomatous tumours than in the pure seminomas too, and this may have contributed to the lymphocytic infiltrate and variability in the lymphocyte density from one area to another.

An interesting finding was that the proportion of T to B cells was very similar (as were the subtype ratios) to those seen in classical seminoma. Of the suppressor/cytotoxic subpopulation, most cells were of suppressor lineage ($CD8^+$ $Leu15^+$). These together with tumour cells were transferrin receptor positive (TR^+) – indicating possible proliferative activity – but very few inflammatory cells expressed I1-2 receptors (an early activation antigen).

Because $CD4^+$ helper T cells need to identify antigens in the context of self Class II MHC antigens, the fact that most tumour cells lacked Class II antigens (DR, DQ) suggests that these cells are incapable of presenting their own antigens directly. These cells would have to shed their tumour antigens in order to evoke an immune response. Further, $CD8^+$ cytotoxic T cells are restricted by Class I antigens such that tumor cells would need to express these Class I antigens to be able to be killed by cytotoxic cells. In the present study a variation in tumour Class I expression was observed.

Most of the tumours in this study were mixed (Table 1). Macroscopically, the specimens submitted for routine histopathology and immunohistology were identical, but microscopically their component germ cell subtypes may have been different since it was impossible to be absolutely sure of the germ cell subcategory in the immunohistological sections. Nevertheless, lower and higher numbers of inflammatory cells did relate to reference histopathological diagnoses in which embryonal carcinoma and malignant teratoma respectively predominated, this being consistent with published reports [2, 10]. The heterogenous nature of these tumours was also evident in the differential expression of Class I and Class II antigens. The relationship between this expression and the functional activity of the infiltrating lymphocytes remains to be ascertained.

Ritchie and El-Demiry reported very few lymphocytes to be present in normal testes [4, 13]. In this study the one overtly normal testis (biopsied at the time of correction of a primary idiopathic hydrocoele and examined by routine, standard, histological techniques as well as by immunohistology) contained only 3 lymphocytes per high power field.

The significance of inflammatory cell infiltrates in germ cell tumours is unknown. The absence of natural killer cells is not unexpected since current opinion identifies their role to be mostly in the circulation rather than in solid tissues [17]. In this study interdigitating cells were few and most macrophages were of phagocytic phenotype. Germ cell tumours are considered to be inherently different biologically to other solid malignancies and the testis is an organ reported to be an immunologically privileged site [6]. Large numbers of inflammatory cells had been attracted to both primary seminomatous and nonseminomatous testis tumours (although much more patchily distributed in the latter) but their degree of immunological activity is as yet unknown. The significance of a lack of I1-2 receptor expression also remains to be ascertained. Nevertheless, an ability to induce and manipulate activation of these cells could prove to be a useful therapeutic mechanism.

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Dr. R. A. Gardiner
Department of Surgery
Clinical Sciences Building
Royal Brisbane Hospital
Herston, 4029
Australia