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Immunopathology of *in situ* seminoma

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Summary. In this study of the seminomatous human testis the composition, activity and apoptosis of lymphocytes infiltrating the immune-privileged seminiferous tubules with *in situ* seminoma were studied by immunohistochemistry and DNA fragmentation detection. Likewise the lymphocytes infiltrating the invasive seminomas were studied. The study showed equal numbers of CD4+ and CD8+ T cells and B cells, about 30% of the cells. Very few T γ/δ and NK cells were present. The activity in terms of IL-2-R, FasL and perforin expression was low. Apoptosis of the lymphocytic cells was limited. No differences were observed between the lymphocytes in seminiferous tubules with *in situ* seminoma and the lymphocytes in invasive tumours. The study suggests that either specifically committed lymphocytes are not present or, if present, immune-suppressing mechanisms in addition to FasL may be working.

Keywords: in situ seminoma, immune privilege, immune surveillance

Concepts of immune surveillance of cancer maintain that tumour-infiltrating lymphocytes (TIL) more or less successfully eliminate tumour cells (Klein 1980; Kradin & Bhan 1993). Cytotoxic T cells are believed to play a major role by recognising tumour-specific peptides presented by major histocompatibility complex (MHC) class I molecules on the surface of tumour cells (Elliot *et al*. 1989; Townsend & Bodmer 1989).

In immune-privileged areas such as the testis where immune reactions are suppressed (Wayne Streilein 1995) the role of immune surveillance of cancer is obscure. In the testis the seminiferous tubules are protected from immune attack by FasL molecules expressed by Sertoli cells, inducing apoptosis in Fas-expressing lymphocytes (Bellgrau *et al*. 1995). The FasL molecules form a layer at the periphery of the tubules located at the foot processes of the Sertoli cells in rodents and humans (French *et al*. 1996; Lee *et al*. 1997; Brændstrup *et al*. 1999).

In seminiferous tubules with *in situ* seminoma (cis) Sertoli cells are eventually displaced to central areas of the cis tubules by the neoplastic cells (Brændstrup 1996). The FasL layer then becomes discontinuous and only sporadic FasL expression can be recognized on the displaced Sertoli cells. This may allow T lymphocytes to infiltrate the cis tubules (Brændstrup *et al*. 1999). Evidently in invasive seminomas Sertoli-cell FasL has no significance. Invasive seminomas are characteristically infiltrated by numerous TIL including many CD8+ T cells (Bell *et al*. 1987; Nakanoma *et al*. 1992; Wei *et al*. 1992; Nouri *et al*. 1993; Torres *et al*. 1997).

However, since the seminoma cells in cis and invasive tumour do not express MHC class I and ICAM-1 molecules which mediate cellular interaction between CD8+ T cells and their targets, the role of the CD8+ T cells in seminomas is not clear (Bell *et al*. 1987; Vanky

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et al. 1990; Nouri *et al*. 1993; Brændstrup 1996; Brændstrup *et al*. 1996). Sertoli cells of cis tubules as opposed to those of normal tubules, on the other hand, express both MHC class I and ICAM-1 molecules and therefore one may speculate that some of the lymphocytes infiltrating cis tubules are autoreactive CD8+ T cells with specificity against the antigens presented by the modified Sertoli cells (Brændstrup 1996; Brændstrup *et al*. 1996). If so, immune activity might be observed in these lymphocytes.

The present study was therefore undertaken to determine the composition, activity and apoptosis of the infiltrating lymphocytes in *in situ* seminomas and to compare these lymphocytes with those of the invasive tumours.

Materials and methods

The material derives from the files of the Department of Pathology, Glostrup Hospital. It consists of 22 cases of seminomas collected from 1988 to 1996 and selected on the basis of technical quality and the presence of cis seminiferous tubules. Blocks containing invasive tumour and surrounding tissue with normal and cis seminiferous tubules were chosen. These specimens had been fixed in formaldehyde and stored as paraffin-embedded blocks. In addition the material consists of frozen tissue stored at −80°C from another 12 consecutive cases of seminomas collected from 1996 to 1999. Specimens from these cases were selected on the basis of technical quality only. Some of these samples contain only tumour or surrounding tissue. Sections from the material were cut and stained with hematoxylin and eosin (H & E) for conventional histology to identify relevant areas for immunohistochemical studies.

Immunohistochemistry

The antibodies used in the study are described in Table 1. As secondary antibodies were used biotinylated rabbit antimouse Immunoglobulins (DAKO A/S, Copenhagen, Denmark; code E 0354, lot 067) and a swine antirabbit Immunoglobulin (DAKO; code E 353, lot 053).

DAKO EnVision + System (DAKO; code 4004) was used as secondary antibody binding to anti-Fas. This includes a horseradish peroxidase-labelled polymer conjugated with the secondary antimouse antibody. Biotinylated rabbit antirat immunoglobulin (DAKO; code E 0467, lot 097) was used as secondary antibody to anti-FasL.

Antibodies to CD3, CD8, CD20, CD79, CD68, CD56, CD57 and S100 were used on formalin-fixed tissue. Antibodies to CD4, perforin, IL-2-R, T γ/δ receptor, Fas and Fas-L were used on frozen tissue.

The avidin–biotin/peroxydase immunohistochemical procedure for formalin-fixed and fresh frozen tissue used has been described previously (Brændstrup 1996; Brændstrup *et al*. 1999). Mayers haemalun was used as counter stain. Endogenous peroxydase was initially blocked by H_2O_2.

Controls

As positive controls were used formalin-fixed and frozen tissue from various organs containing lymphoid tissue

Table 1. Primary antibodies used for immunohistochemistry

Antihuman antibody	Specificity	Dilution	Source
Rabbit anti T-cell (*p*)	CD3	1/50	DAKO, code A0452, lot 035
Mouse anti helper T-cell	CD4	1/100	DAKO, code M0716, lot 105
Mouse anti cytotox./supp. T-cell	CD8	1/500	DAKO, code M7103, lot 045
Mouse anti B-cell	CD79α	1/25	DAKO, code M7050, lot 107
Mouse anti B-cell	CD20	1/400	DAKO, code M755, lot 083
Mouse anti NCAM-1 (NK-cells)	CD56	1/500	NeoMarkers, cat# MS-204-P1, clone 123C3.D5
Mouse anti HNK-1/leu7 (NK-cells)	CD57	1/1000	Zymed, cat# 18–0167, lot 80140246
Mouse anti macrophage	CD68	1/1000	DAKO, code M876, lot 084
Rabbit anti S-100* (dendritic cells) (*p*)	S-100 A & B	1/1000	DAKO, code Z0311, lot 129
Mouse anti pan γ/δ T cell	γ/δ T-cell receptor	1/25	T-cell Diagnostics, cat#TCR 1061, lot 302867
Mouse anti interleukin-2 receptor	CD25	1/200	DAKO, code M0731, lot 025
Mouse anti perforin	Perforin	1/100	Ancell, cat#358–020, lot 9718001
Mouse anti Fas	CD95	1/150	DAKO, code M3554, lot 066A
Rabbit anti Fas Ligand	CD95L	1/50	Alexis, code 804–124-C100, clone Mike-1

Antibodies were monoclonal except for those marked (*p*), * anticow S-100 crossreacts with human S-100.

such as tonsils, small intestine and spleen. For negative controls the specific antibodies were omitted.

Apoptosis

A DNA fragmentation kit was used, Klenow-FragEL™ (Oncogene Research Products, Cambridge, MA, USA/ Calbiochem, cat# QIA21). In this kit DNA polymerase I adds biotin-labelled and unlabeled deoxynucleotides to the ends of DNA fragments generated during apoptosis. Streptavidin-horseradish peroxidase and diaminobenzidine were used as a detection system. Methyl green was used as a counterstain. Endogenous peroxidase was blocked by H_2O_2. This system was used on the paraffin-embedded material and deparaffinization and rehydration were initially performed. Intestinal mucosa was used as a positive control. For negative controls the labelling step was omitted and the slides kept in reaction buffer.

Scoring of stained cells

Each slide was labelled at random with ink dots using a metal grid. In each slide cis tubules with and without lymphocytic infiltrates were identified and enumerated. In the invasive tumours, areas which happened to be located on one side of the ink dots inside a square in an ocular grid were selected for counting. In the cis tubules with lymphocytic infiltrates all lymphocytes and all labelled lymphocytes were counted. In the selected tumour areas approximately 500 lymphocytes including labelled and unlabelled lymphocytes were counted. The percentages of labelled lymphocytes were calculated. Each of the two observers (BB and LJ) examined half of the material.

The inter- and intra-observer variations were estimated separately. In each of 25 randomly chosen slides a given area (square) was counted twice by both observers. The percentages of labelled lymphocytes were determined. The Spearman rank–order correlation coefficient θ was used to analyse inter- and intra-observer variation. The Mann–Whitney rank sum test was used to examine differences in percentages of labelled cells between *in situ* and invasive seminomas.

Results

Varying numbers of cis tubules were infiltrated by lymphocytes, the average being 15% of the cis tubules. The degree of inflammation in these tubules varied from a few lymphocytes to heavy concentric infiltrates invading the epithelium (Figures 1–4). Normal seminiferous tubules were devoid of inflammatory cells.

The results of the immunohistochemistry are presented in Table 2. As will be seen, great variation in the

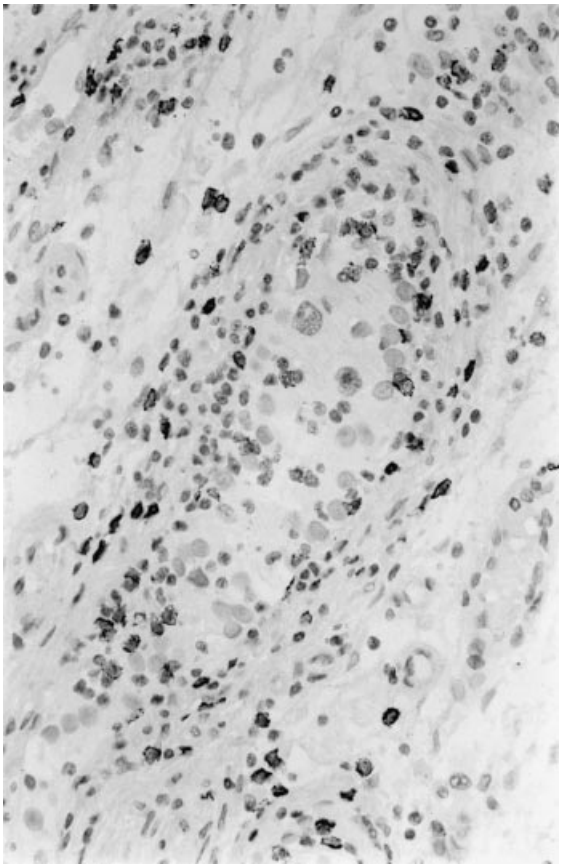

Figure 1. Immunohistochemical staining for CD8+ T cells in and around cis seminiferous tubule. Positive cells have dark granules along the cell membrane. ×400.

percentages of labelled cells was observed, as illustrated by the ranges. Only minor differences between the mean percentages of stained cells in the cis tubules and the tumours were seen. These differences were significant only for T γ/δ cells, CD 68 and FasL. CD8+ T cells (Figure 1), CD4+ T cells and B cells (Figure 2) were present in equal numbers around one-third of the cells. As a 'control' of T cells the number of CD3-expressing T cells corresponded to the sum of CD8 and CD4 cells. B cells were demonstrated by CD20 as well as CD79, the latter showing more positive cells in accordance with the broader range of B cells expressing this marker. Some of these cells had a plasmocytoid appearance.

CD56-, CD57-, IL-2-R-, Perforin- and FasL-expressing cells each comprised a few per cent of the inflammatory cells.

About 25% of the cells expressed Fas. CD68 expressing cells, i.e. macrophages, were seen in substantial numbers (Figure 3) with relatively more positive cells inside the tumours.

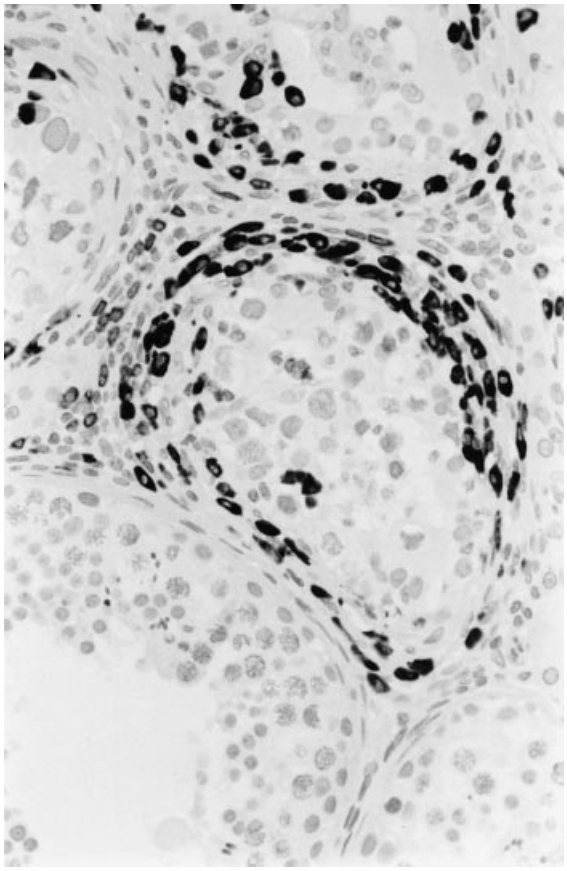

Figure 2. Immunohistochemical staining for CD79+ B cells in and around three cis seminiferous tubules. One normal seminiferous tubule is present, lower part. ×400.

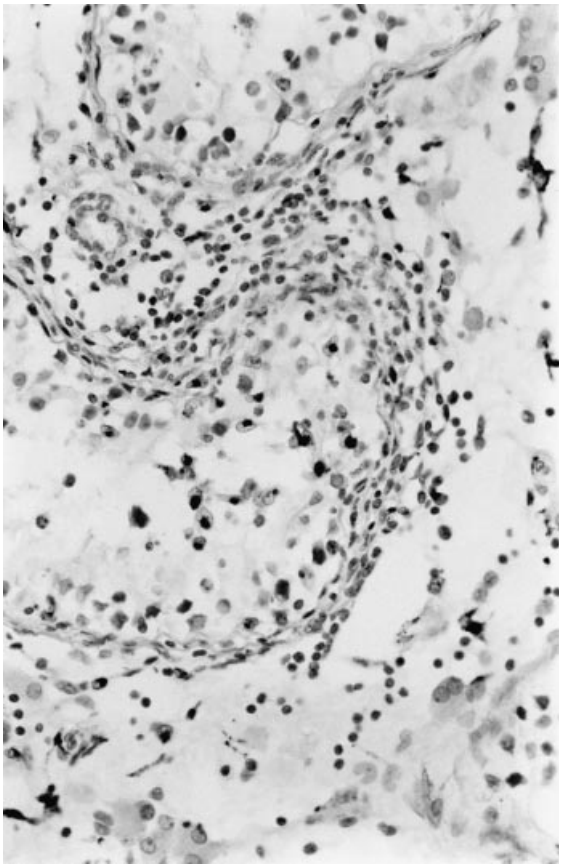

Figure 3. Immunohistochemical staining for CD68 positive macrophages seen as dark cells in and around a cis seminiferous tubules. ×400.

S-100 positive dendritic cells were observed in the cis tubules with lymphocytic infiltrates (Figure 4), the number of cells per cross-section was 0.7. Dendritic cells were present among lymphocytes in the tumours, the average number per high power field (×400) was 5.2 cells.

The number of apoptotic lymphocytes in and around cis tubules was 0.61% and inside tumours 0.91%. Apoptosis was observed in only a few tumour cells in the seminomas and no identifiable Sertoli cells were observed in the tubules studied.

The inter- and intra-observer variations were low, Spearman's θ: BB/LJ: 0.8853–0.9269, BB/BB: 0.9746 and LJ/LJ: 0.9396.

Discussion

In seminomas TIL and tumour cells are separated creating an almost morphologically biphasic pattern. In contrast the peri- and intra-tubular lymphocytes of a number of cis tubules gives an impression of an inflammatory process targeted at the content of these cis tubules (Figures 1–4).

The present study showed that CD8+ T cells, CD4+ T cells and B cells each constituted about one-third of the lymphocytic infiltrates whether attacking cis tubules or being TIL in the seminomas.

In previous studies which have focused on lymphocytes inside the infiltrating tumour only, CD8+ T cells were the dominating cells (Bell *et al*. 1987; Nakanoma *et al*. 1992; Wei *et al*. 1992; Nouri *et al*. 1993; Torres *et al*. 1997). Only one additional study found as many B cells in the tumours as shown here (Nakanoma *et al*. 1992). Autoimmune antibodies towards the germinative epithelium of cis tubules has been reported (Lehmann & Müller 1987) and a humoral immune response is induced in the mouse testis used for induction of experimental teratoma (Sundström *et al*. 1999). These observations

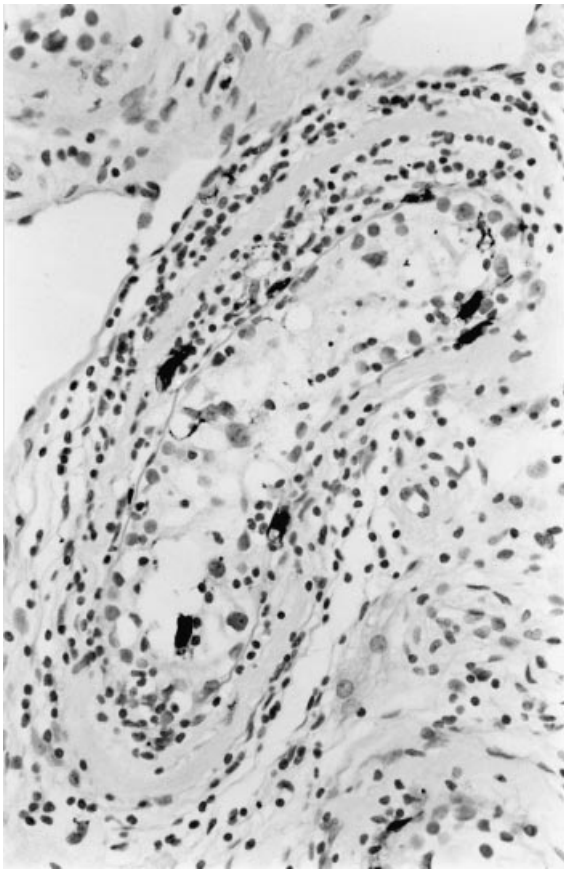

Figure 4. Immunohistochemical staining for S-100 positive dendritic cells seen as strongly stained cells in and around a cis seminiferous tubule. ×400.

may be pertinent to the many B cells in the cis tubules in the present study.

The few IL-2-R-expressing lymphocytes in cis tubules and the tumours and the only occasional presence of lymphocytes expressing perforin and FasL molecules used by cytotoxic T cells to kill target cells (Lowin *et al*. 1994; Lynch *et al*. 1995) do not support substantial immune activity although more FasL positive cells were observed in cis tubules. Furthermore very little apoptosis of the lymphocytes was observed.

Previous studies of TIL inside seminomas have shown activity of TIL in terms of *in vitro* responsiveness to IL-2 and MHC class II expression (Nouri *et al*. 1993) and also a considerable number of Fas-expressing lymphocytes (Brændstrup *et al*. 1999). This may merely reflect the propensity of such activated lymphocytes to randomly pass vessel walls expressing relevant adhesion molecules (Carlos & Harlan 1994). ICAM-1, VCAM-1 and ELAM-1 are expressed on endothelial cells in seminomas (Brændstrup *et al*. 1996).

NK cells and T γ/δ cells, believed to be part of an early response to tumours, seem to play no significant role even though seminoma cells without MHC class I molecules would seem to be ideal targets for NK cells (Ljunggren & Kärre 1986; Mak & Ferrick 1998). A study of 22 seminomas confirms our data of T γ/δ cells (Nouri *et al*. 1993) while a study of three dysgerminomas and one seminoma found increased numbers of T γ/δ cells compared to peripheral blood (Zhao *et al*. 1995).

The absence of MHC class I molecules and ICAM-1 on seminoma cells is not compatible with conventional concepts of a direct cytotoxic T-cell attack on tumours and this is consistent with the limited immune activity of the TIL.

Mouse Sertoli cells show adhesion of lymphocytes

Table 2. Immunohistochemistry and apoptosis of lymphocytes in carcinoma *in situ* and seminoma

Per cent of lymphocytes (range)

	CD8	CD4	CD3	CD20	CD79	CD25	CD68
Cis	32.2 (18.1–56)	33.6 (18.4–49.4)	63.9 (54.2–76.7)	18.8 (0.8–27)	35.6 (14.5–59)	1.2 (0–4)	4.9 (0–16.6)
Tumour	35.8 (9.9–74)	27.9 (1.2–42.7)	68.8 (37.8–90)	15.9 (1.4–33.9)	31 (5.3–44.6)	1.6 (0–3.3)	13.2 (6.2–17.5)
	CD56	CD57	T γ/δ	Perforin	FasL	Fas	Apoptosis
Cis	<0.1	1.4 (0–6.1)	2.0 (0.8–5.1)	1.3 (0–6.8)	2.0 (0.8–3.1)	24.7 (85–33)	0.6 (0–2.5)
Tumour	<0.1	1.4 (0.7–1.9)	5.0 (0–15.7)	3.3 (0–10.3)	0.8 (0.1–1.3)	19 (5–48)	0.9 (0.3–2.5)

The Mann–Whitney rank sum test showed significant difference for CD68 and Tγ/δ ($P<0.05$) and for FasL ($P<0.01$).

and expression of ICAM-1 *in vitro* when exposed to IL-1, TNF-alfa and IF-gamma and it has been suggested that such Sertoli cells, normally being immunosuppressive, could contribute to autoimmunity in the testis (Riccioli *et al*. 1995).

In the cis tubules the low apoptosis/activity of the infiltrating lymphocytes may be explained by the reduced expression of FasL on the Sertoli cells (Brændstrup *et al*. 1999), and/or the absence of lymphocytes with specificity to the tubular content and/or absence or insufficient numbers of the relevant stimulatory molecules.

Dendritic cells, present in cis tubules and tumours, can transport phagocytozed apoptotic tumour cells and other cells from the periphery to the draining lymph nodes and give rise to clonal expansion of lymphocytes with specificity to antigens from the apoptotic cells (Grabbe *et al*. 1995; Albert *et al*. 1998). In this way the dendritic cells in seminomas and cis could prime CD4+ T cells by conventional MHC class II pathway and CD8+ T cells by cross-presentation of exogenous Sertoli cell and tumour cell peptides by MHC class I molecules on DCs (Kurts *et al*. 1996; Rock 1996; Albert *et al*. 1998).

These lymphocytes could be among those in the seminomas unable to recognize the target cells. The absence of an immune reaction with subsequent apoptosis of the lymphocytes may explain the large numbers of lymphocytes which accumulate in seminomas.

In summary, even though the immune privilege of the testis in terms of FasL protection is disturbed during development of neoplasia, only low CD8+ T cell activity in terms of expression of perforin, FasL and apoptosis of the lymphocytes infiltrating the cis tubules can be recognized. The same low activity was observed in TIL in the invasive seminomas. This may indicate that either no lymphocytes with specificity towards tumour cells or Sertoli cells are present or, if present, the lymphocytes are unable to react to or recognize the target cells. In the case of the seminoma cells this can be explained by the negative expression of MHC class I and ICAM-1 molecules. In the case of Sertoli cells in the cis tubules, immune-suppressing mechanisms alternative to FasL may be acting.

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