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Immunohistochemical characterization of transplantable rat squamous cell carcinoma (FF-6) in skin and thymus

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Summary. FF-6 is a transplantable squamous cell carcinoma which originally arose in the facial skin of a DA rat. It was established after maintaining the tumor in the subcutaneous tissue or peritoneal cavity of DA rats conventionally for over 30 generations. When the soybean-sized original FF-6 tumor was transplanted subcutaneously, it became an oval, hard, whitish, solitary and thumb-head-sized nodule within one month. After intraperitoneal transplantation of FF-6, it formed many nodules ranging from miliary to thumb-head size, which adhered and/or metastasized to many abdominal organs. When FF-6, cut into small pieces, was injected into the lower lip, the tumor grew bigger *in situ*, and metastasized to regional lymph nodes. Histologically, FF-6 was characterized as a well-differentiated squamous cell carcinoma, showing positive staining with anti-keratin, anti-laminin, anti-collagen type IV, anti-fibronectin and UB-14 antibodies.

This transplantable tumor may be useful for analyzing the mechanisms of proliferation and metastasis of squamous cell carcinoma *in vivo*, and the host defence mechanism in rats, as well as being a suitable model of human squamous cell carcinoma.

Key words: Squamous cell carcinoma, Rat transplantable tumor, FF-6, Monoclonal antibody, Immunohistochemistry

Introduction

Despite extensive research on the diagnosis, therapy and prevention of malignant neoplasms world wide, neoplasm is still the leading cause of human death. Animal models are necessary for analyzing the effects of drugs and their side effects in malignant states, for clarifying the mechanism of metastasis and for developing more effective methods of diagnosis. There have been various reports on chemical carcinogenesis in murine models, concentrating on the routes of administration, drug volume and treatment period (Tatematsu et al., 1991; Tanaka et al., 1992), differences in susceptibility to carcinogens among rat strains (Kitano et al., 1992), and gene mutations in carcinoma cells (Yokota et al., 1987; MacKay et al., 1988; Makino et al., 1992). Since Rygaard and Povlsen (1969) reported heterotransplantation of a human malignant tumor in nude mice, this technique has been used in many experimental fields including analysis of tumor metastatic routes (Hata et al., 1978; Ueyama et al., 1978), since such transplanted tissues maintain their human tumor form over a long period *in vivo*. Further applications have included the establishment of tumor cell lines *in vitro*, cells of which have been used as sources for examining the production of certain proteins (Nakamura, 1991), and also susceptibility to anti-tumor drugs (Nishihira et al., 1979; Simms et al., 1980; Matsuoka et al., 1987; Yano et al., 1988). However, nude mice are immunodeficient animals that lack T-lymphocytes, making it difficult to determine the effects and side effects of drugs on them, and routes of metastasis. There is a further problem in that *in vitro* data may not always reflect the mechanism *in vivo*. On the other hand, there is a scarcity of reports about maintenance of spontaneous solid malignant tumors *in vivo* for long periods.

In the present paper we describe the establishment *in vivo* of a spontaneous well-differentiated rat squamous cell carcinoma (SCC), FF-6, and its isogeneic transplantation for over 30 generations under conventional conditions. Immunohistologically, it was shown to contain laminin, keratin, collagen type IV and fibronectin, like normal squamous cell epithelium, but did not express major histocompatibility complex (MHC) class I antigen.

From an immunological view point, thymus is a central organ of the immune system, being a site of T-cell maturation and distribution to the peripheral circulation. Ontogenically, parts of the thymus are derived from endodermal and ectodermal epithelial

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components (Cordier and Haumont, 1980). In fact, Naylor et al. (1988) reported that among 192 primary thymic tumors in 4281 rats examined for chemical toxicity and carcinogenicity, 11 were SCC. However, there have been few reports on the influence of thymic epithelial tumors on T-cell production in the thymus and immune system. Therefore, we examined the effects of FF-6 SCC on immune organs and peripheral blood after transplantation of small tumors into the thymus.

Materials and methods

Animals

Inbred DA (RT-1^a) and Lou/M (RT-1^u) rats 2-4 months old were maintained conventionally at the Institute of Laboratory Animals, Yamaguchi University School of Medicine. All the rats were housed 3 or 4 to a plastic cage on wood-chip bedding, and maintained in an air-conditioned room at a controlled temperature (25 ± 2 °C). They were fed with a standard diet of Oriental MNF (Oriental Yeast Co., Tokyo, Japan).

FF-6 maintenance and passage

FF-6 carcinoma was a spontaneous tumor that arose originally in the facial skin of a 24-month-old male DA rat, which had been maintained conventionally at the Institute of Laboratory Animals, Yamaguchi University School of Medicine. The tumor was maintained in the peritoneal cavity or subcutaneous tissue between the bilateral scapulae of DA rats.

A soybean-sized piece of the solid FF-6 tumor was transplanted into the subcutaneous space (ISC) or peritoneal cavity (IP). In addition, about 0.1 mg of FF-6 was cut into small pieces and injected into the lower lip (IL) or thymus through an 18 G x 1-1/2" needle.

Antibodies

Various kinds of mouse ascitic-type monoclonal antibodies against rat and human antigens were used. OX-2, OX-3, OX-6 (McMaster and Williams, 1979), OX-7 (Mason and Williams, 1980), OX-18 (Fukumoto et al., 1982), W3/13, W3/25 (Williams et al., 1977), MAS-251 and MAS-252 (Haynes, 1981) were from Sera-lab Ltd (Crawley Down, Sussex, UK). HAM-2 (Fukumoto et al., 1984), HAM-4 (Tamakoshi et al., 1985), HAM-8 (Fujikura et al., 1993), UB-13 and UB-14 (Tomonaga et al., 1990) hybridomas were maintained in our laboratory. MoAb-27 (Takeshima et al., 1990) was generously provided by Dr. H. Kato. To clarify the origin of FF-6, rabbit anti-laminin, anti-keratin, anti-fibronectin and anti-collagen type IV polyclonal antibodies, obtained from Medac Gesellschaft für Klinische Spezialpräparate GmbH (Hamburg, Germany), were used.

Fluorescein isothiocyanate (FITC)-conjugated OX-19 directed against rat pan-T cells and FITC-conjugated W3/25 against rat helper T cells were from Sera lab. FITC-conjugated OX-8 against rat T suppressor/cytotoxic cells was from Serotec (Oxford, UK). They were used for direct immunostaining to analyze T-cell subsets in peripheral blood by flow cytofluorometry using an EPICS 753 (Coulter Corporation, Hialeah, FL, USA). The specificities of the antibodies are summarized in Table 1.

Indirect immunohistochemical staining

Rats carrying FF-6 carcinoma were anaesthetized with diethyl ether and then sacrificed by exsanguination. Thymus, lung, liver, kidney, pancreas, spleen, lymph node and FF-6 tumor tissues were removed immediately and fixed with Carnoy's solution. The tissues were embedded in paraffin and stained with haematoxylin and eosin after thin-sectioning. Samples of tumor and some tissues were embedded in O.C.T. compound (Miles, Elkhart, IN, USA), rapidly frozen, and sectioned at a thickness of 6 μm on a cryostat (Bright OT/FAS, UK) at -25 °C. Frozen sections were air-dried for 30 min at room temperature and immersed in ice-cold acetone/methanol (1:1) for 10 min for fixation. After washing in ice-cold phosphate-buffered saline (PBS) three times, these specimens were incubated with 100 μl of first-layer antibodies or a control antibody at room temperature for 1 h in a humid chamber, and then left for 10 min at 4 °C. They were washed similarly again and incubated with 50 μl of horseradish peroxidase-

Table 1. Mono- and polyclonal antibodies used in this study.

ANTIBODY	CLASS AND SUBCLASS	RECOGNITION
OX-2	IgG1	rat thymocytes, brain, B-cells
OX-3	IgG1	rat Ia polymorphic
OX-6	IgG1	rat Ia common non-polymorphic
OX-7	IgG1	rat Thy-1
OX-18	IgG1	rat major histocompatibility complex class I
W3/13	IgG1	rat thymocytes, T-cells, brain, plasma cells
W3/25	IgG1	rat CD4 (helper-T), most thymocytes, macrophages
MAS-251	IgG2	human thymic epithelium
MAS-252	IgM	human subcapsular cortical and medullary endocrine epithelium
HAM-2	IgG2a	rat major histocompatibility complex class I different epitope from OX-18
HAM-4	IgG1	rat liver, kidney, submandibular gland, etc
HAM-8	IgG2a	gap junction of rat and mouse
MoAb-27		human squamous cell carcinoma
UB-13	IgM	various organs of skate, frog, turtle, chicken and rat
UB-14	IgG2a	thymus and brain of skate
anti-laminin		glycoprotein in basement membrane
anti-type IV collagen		type IV collagen
anti-keratin		cytoskeleton and keratin in epidermis, etc
anti-fibronectin		fibronectin in interstitium

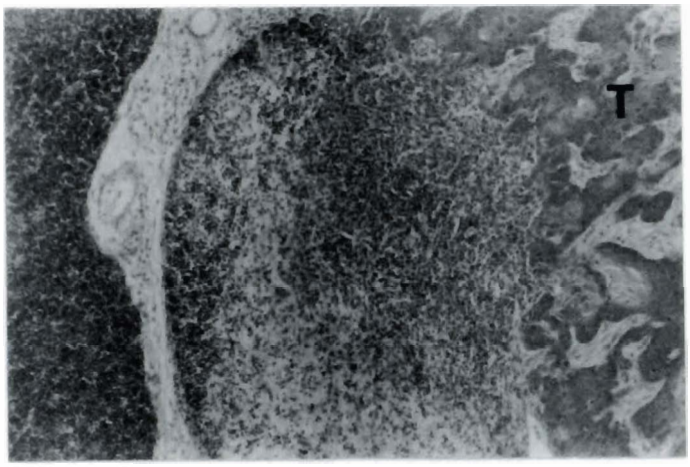

Fig. 1. Paraffin-embedded thin section stained with haematoxylin and eosin. x 70. Tumor tissue (T) has invaded the thymic lobule, and the cortico-medullary structure of the lobule is destroyed.

conjugated goat F (ab')$_2$ anti-mouse IgG containing 5% heat-inactivated normal rat serum for 1-2 h at 4 °C in a humid chamber. All antibodies diluted with PBS were used at a concentration of 3-50 µg/100 µl/tissue. After washing three times in ice-cold PBS, the sections were developed for 5 to 7 min at room temperature with 100 µl of substrate buffer (0.025 M Tris-HCl buffer, pH 7.6, containing 0.05% 3,3'-diaminobenzidine, and 0.1% H_2O_2) and serial sections were stained with haematoxylin.

Results

FF-6 transplanted into the subcutaneous region, peritoneal cavity and lower lip

FF-6 tumors transplanted subcutaneously between the bilateral scapulae, showed gradual enlargement *in situ*. After about 3 to 4 weeks, the tumor nodule formed a slightly haemorrhagic ulcer, when it was usually 2 to

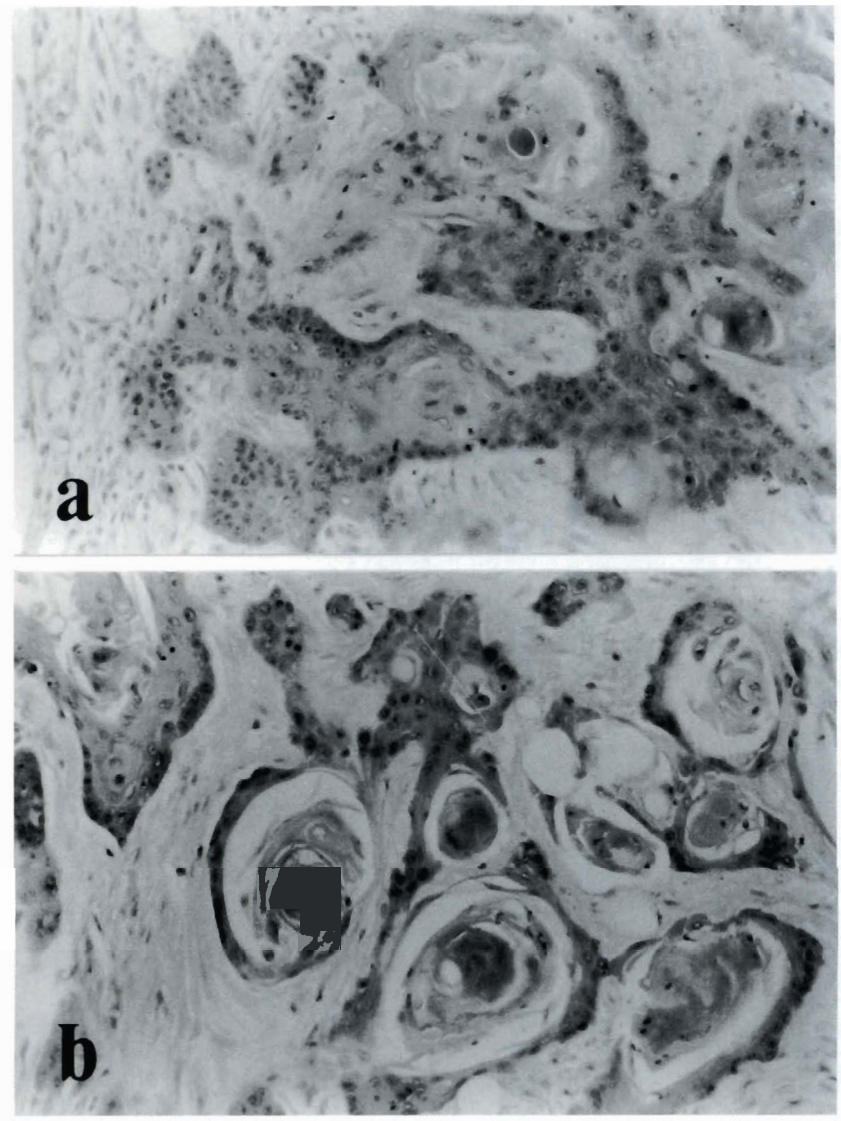

Fig. 2. Paraffin-embedded thin section stained with haematoxylin and eoxin. x 135. **a.** FF-6 tumor is encapsulated with simple columnar or cuboidal epithelium, and a relatively thick fibrous layer is recognizable beneath the capsule. The fibres extend to the centre of the tumor and separate it into many discrete tumor areas. **b.** The outermost layer consists of basal cells in continuity with polyhedral cells, squamous cells, and a keratinous layer toward the centre. Cancer pearls are recognizable in some areas. The fibres in the interstititum forms regular arrangements of connective tissue surrounding the tumor foci. The cellular component, mainly fibroblasts, is sparse in the interstitium.

2.5 cm in diameter. The neoplasm adhered to the muscle tissue under the growth site about 2 weeks later. When the host was killed 1 to 2 months later, no metastatic focus was recognizable in the regional lymph nodes macroscopically or microscopically.

One month after transplantation of a small piece of FF-6 into the peritoneal cavity, a large number of tumor masses ranging from miliary to thumb-head-sized were recognized in the peritoneal cavity. The tumor masses were hard, whitish, and globe- or egg-shaped, and accompanied by many arteries. Large nodules were relatively soft because of central necrosis. Scattered metastatic nodules were attached to the peritoneum, omentum and mesentery. Occasionally, they invaded the abdominal wall muscle, diaphragm muscle and some organs such as the liver, spleen, kidney, pancreas and lymph nodes. Upon cutting the diaphragm with a razor, some tumor masses were recognized on the bilateral sides, but they were not attached to the lungs. The nodules adhered to abdominal muscle, but were not seen in the subcutaneous tissue. In the liver, spleen and kidney, the tumor masses were usually visible through the capsule, but were rarely present at deep sites in the liver as small nodules.

When small pieces of FF-6 were injected into the centre of the lower lip, the tumor grew into a mass 1 to 1.5 cm in diameter *in situ* within 2 to 3 weeks. When the host became unable to take food due to enlargement of the tumor, no metastatic foci were recognizable in the cervical lymph nodes macroscopically, but well-differentiated SCC tissue was evident in the regional lymph nodes microscopically.

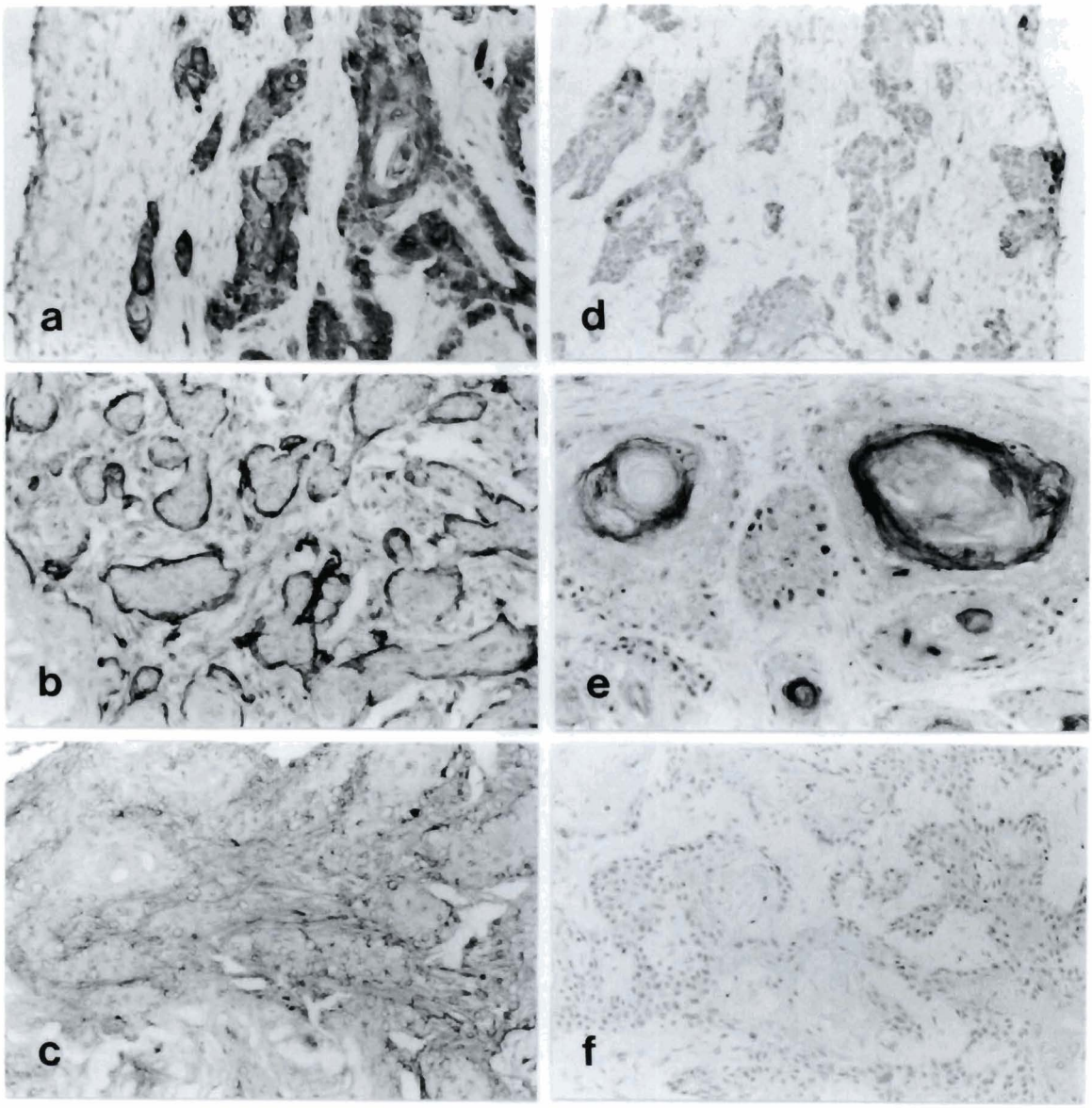

Fig. 3. Frozen 6-µm-thick sections of FF-6 tumor stained immuno-histochemically using anti-keratin **(a)**, anti-laminin **(b)**, anti-fibronectin **(c)**, MAS-252 **(d)**, UB-14 **(e)**, and control **(f)** antibodies (see text). x 90

Table 2. Cross-reactivity of mono- and polyclonal antibodies against rat SCC.

ANTIBODY	CROSS-REACTIVITY IN RAT SCC		
	Horny layer	Basal layer	Basement membrane and interstitium
OX-2	±	±	- (capillary ++)
OX-3	-	+++	-
OX-6	-		+ (some fibrous cells)
OX-7	-	±	
OX-18	-	-/± (cytoplasm)	
W3/13	-	++ (nucl. memb.)	
W3/25	+	±	
MAS-251	-	+ (nucl. memb.)	
MAS-252	+/++	+/++	-
HAM-2	-		-
HAM-4	-		-
HAM-8	-	-	
MoAb-27	-	-	-, + (fibrous)
UB-13	-	-	
UB-14	+++		-
anti-laminin	-	+++	+++
anti-type IV collagen	-	+++	+++
anti-keratin	+++	-/±	-
anti-fibronectin	-	+++	+++

Degree of antigen expression was shown as follows: -, negative; ±, weakly positive; +, positive; ++, strongly positive; +++, very strongly positive.

FF-6 transplanted into thymus

The thymus into which the tumor was injected showed a macroscopic nodule 2 to 3 weeks later, but no metastases to parathymic lymph nodes were observed. The tumor enlarged thereafter, but the growth rate was less than that of FF-6 transplanted ISC or IP. The maximum diameter attained was about 2 cm at 10 weeks. Sometimes, metastasis was evident histologically in regional lymph nodes after 3 weeks. The histological features of FF-6 in the thymus and lymph nodes were the same as those of tumors in the peritoneal cavity. The lobule and cortico-medullary structure of the thymus was destroyed in the region adjacent to the tumor (Fig. 1), and some eosinophilic cell infiltration was evident. Sometimes, duct-like structures were newly formed within 1 to 10 weeks in the subcapsular region, cortex and medulla of the remaining thymus, whose epithelium consisted of 2 to 3 layers of columnar cells. There was homogeneous eosinophilic material in the ducts (data not shown). The maximum diameter of the ducts was less than 500 μm. These structures were also sometimes observed in the thymus of rats given a sham operation, but they were smaller than those in the thymus containing transplanted tumors.

Histological and immunohistochemical findings

FF-6 was a well-differentiated SCC showing a relatively normal maturation sequence from small basal cells at the periphery adjacent to the stroma along with small blood vessels, to squamous epithelium closer to the centre of epithelial nests (Fig. 2a). Cancer pearls were seen as areas of central keratinization surrounded by squamous cells (Fig. 2b). Mitosis was also evident in the basal cell layer. The central parts of large tumors over 1 cm in diameter occasionally showed necrosis (data not shown).

The basement membrane of the tumor was stained positively with anti-laminin (Fig. 3b), anti-fibronectin (Fig. 3c) and MAS-252 (Fig. 3d) antibodies. The keratinous layer was stained positively with anti-keratin (Fig. 3a), MAS-252 and UB-14 (Fig. 3e) antibodies, and this area corresponded to an eosinophilic layer after staining with haematoxylin-eosin. Anti-keratin polyclonal antibody reacted strongly with tumor cells and some keratinous layers in the centres of tumor foci, but the interstitium was negative (Fig. 3a). Anti-laminin antibody strongly stained a monolayer in the interstitium just beneath the basal cell layer. Fine, laminin-positive fibres were recognized sporadically in the interstitium (Fig. 3b). The staining pattern produced by anti-fibronectin antibody was similar to that produced by anti-laminin antibody. The fibrous positively-stained material in the interstitium was more abundant than the laminin-positive material (Fig. 3c). The staining pattern produced by MAS-252 monoclonal antibody was similar to that produced by anti-keratin antibody, but strongly positive staining was observed on intercellular material between neighbouring basal cells or at the basal cell surfaces. The polyhedral cell layer was weakly positive or negative, but the keratinous layer and interstitium were unreactive with MAS-252 antibody (Fig. 3d). UB-14 monoclonal antibody reacted with keratinous layers in tumor foci, which formed stratified structures. Other areas of the tumor were not reactive with UB-14 (Fig. 3e). Some short fine fibres in the interstitium were stained with MoAb-27, reactive against human SCC (data not shown). There were sporadic spindle- or oval-shaped cells in the interstititum, and about 1/3 of these

were stained with OX-6 (anti-rat MHC class II antibody). However, antibodies against rat MHC class I and gap junction connexin-32 were not reactive with any components of FF-6 (data not shown). No staining was seen in control sections (Fig. 3f). Cross-reactivities of the antibodies used are summarized in Table 2.

Discussion

FF-6 is a spontaneous SCC which arose originally in cheek skin of a DA rat. The tumor was maintained *in vivo* subcutaneously or in the peritoneal cavity of rats maintained under conventional conditions. FF-6 was serially transplantable in this way for over 3 years, and was maintained for over 30 generations. Although an attempt was made to culture this tumor *in vitro*, it was not possible to support its growth in RPMI-1640 medium containing 10% foetal calf serum in the absence of epidermal growth factor (data not shown). FF-6 is considered to be a well-differentiated SCC, since it has many typical components such as basal cylindrical cells, polyhedral cells, squamous cells and fibroblasts in the interstitium, and also interstitial components such as laminin, keratin, fibronectin and collagen type IV. In general, it has been reported that undifferentiated malignant tumors from humans are much easier to transplant into nude mice than well-differentiated tumors, and that tumors with a large amount of interstitium are more difficult to transplant than those with less interstitium (Hata et al., 1978; Ueyama et al., 1978). FF-6 is a well-differentiated SCC with a large amount of interstitium. Many attempts have been made to transplant human malignant tumors into immunodeficient mice and rats in order to examine routes of the metastasis (Hata et al., 1978), or the effects of anti-tumor drugs *in vivo* (Pratesi et al., 1990). However, such methods have drawbacks because the tumor cells are from a separate donor and nutrition is from the host, and furthermore, fibroblasts in the matrix of the tumor tissue may be transformed (Goldenberg and Pavia, 1982), and factors released from the tumor cells affect the host organs (Asano et al., 1980). Therefore, the use of transplantable FF-6 in rats is a very useful method for examining routes of metastasis and the effects of drugs, and determining the operation time to rescue from human malignancies clinically.

The immunohistochemical and histological characteristics of FF-6 were of interest, since the tissue did not express MHC class I antigen, and the parenchyma contained few cells positive for MHC class II antigen, as revealed using HAM-2 and OX-6 monoclonal antibodies. Kärre (1991) reported that MHC class I antigen was not detectable in tumor tissue, whereas MHC class II antigens were demonstrable on malignant cells despite their absence on normal cells (Houghton et al., 1982, 1984; Bernard et al., 1985; Kamma et al., 1991). Furthermore, MHC class II-positive leukocytes were reportedly increased in tumor tissue (Steerenberg et al., 1990; Kamma et al., 1991). when the immunosurveillance system is disturbed by manipulation, producing a graft-versus-host reaction, the epidermal layer of the skin and the epithelium of the small and large intestine express large amounts of Ia antigen (Mason et al., 1981). Similarly, in our study, MHC class I antigens were not expressed in tumor tissue, and only a few MHC class II-positive cells were recognized in the interstitium. We further investigated whether MHC class I-negative FF-6 tissue was transplantable to other rat strains or mice. The results showed that the tumor was able to survive in Lou/M rats but not in BALB/c mice (data not shown).

It is well known that TA-4 is a tumor-associated antigen from human SCC of the uterine cervix (Kato et al., 1987), being present in the serum of patients bearing such SCC (Kato and Torigoe, 1977), and is an important marker for diagnosis and follow-up (Kato et al., 1984). When SCC of the uterine cervix or skin was stained immunohistochemically using MoAb-27, which was raised against TA-4 antigen, the basal cell layer of the tumor tissue was stained (Kato et al., 1987; Matsuta et al., 1987). In the case of FF-6, fine fibres were stained only in the interstitium, and not in the parenchymal region. This discrepancy might be due to a difference between human and rat SCC in the antigenic epitopes recognized by MoAb-27. We are now studying whether there are common antigens in the two tissues using radioimmunoassay. Although there are many reports of monoclonal antibodies reactive with human SCC (Fantozzi, 1991; Battifora et al., 1992), these antibodies recognize tumor-associated, but not tumor-specific, antigens. We are now analyzing whether a tumor-specific antigen is present on FF-6 using anti-FF-6 mouse polyclonal antibody absorbed with normal rat skin, and also employing a UB-17 monoclonal antibody reactive with both FF-6 and human SCC (Inoue et al., in preparation).

The thymus plays an important role as a central immune organ and an inducer of immunological tolerance. It is able to do this because thymic vessels have numerous tight junctions between their endothelial cells, and there is a special anatomical and physiological barrier known as the blood-thymus barrier. Thus, when antigens enter the thymus through this barrier, a different immune reaction might be expected. In fact, transplantation of rat islet allografts into the thymus is a useful method for rescue from, or prevention of diabetes in rats (Posselt et al., 1990; Brayman et al., 1992; Koevary and Blomberg, 1992). We have reported previously that intrathymic injection of whole blood suppressed the cellular immune reaction in Lewis rats for a long period (Hamano et al., 1991). The thymus is a site of T-cell maturation and, like the skin, has components of ectodermal origin; thus there is an important relationship between the thymic epithelium and the proliferation and maturation of T-lymphocytes (Haynes, 1981). Naylor et al. (1988) carried out a pathological study of 192 primary tumors of rat thymus, and reported that, among them, there were 11 SCCs and

one case of small cell undifferentiated SCC. However, no study has yet examined the effects of thymic epithelial tumors on thymocyte production, the immune system and immunological tolerance. In the present study, therefore, we transplanted small pieces of FF-6 into the thymus of young adult rats and examined the effects of the transplanted SCC on the immune system, including the peripheral blood, spleen, and thymus. Extramedullary haematopoiesis and haemosiderosis were seen in the spleen after 2 and 4 weeks, respectively. The body weight of the hosts remained unchanged or decreased gradually, whereas that of non-treated rats and rats given a sham operation gradually increased. The spleen weight of rats bearing the thymic tumors had increased 1 week later, decreased at 2 weeks, and then increased again for up to 6 weeks. The number of erythrocytes in the peripheral blood decreased, and the T/B cell ratio decreased, although the $CD4^+/CD8^+$ cell ratio did not change, as revealed by flow cytofluorometry (data not shown). Few previous studies have examined the peripheral blood data of patients with thymic cancer. However, values of T/B and $CD4^+/CD8^+$ have been reported to vary in some kinds of malignancy (Onsrud et al., 1992; Kaver et al., 1992).

We are now attempting to characterize the cell surface molecules of FF-6 cells, and examining a monoclonal antibody (UB-17) raised against FF-6, which shows interesting cross-reaction with sites common to both rat and human tissues (Inoue et al. in preparation).

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