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# Experimental Mouse Model with Tongue Cancer Produced by KLN-205 Cell Line Inoculation for Development of Tumor Vaccine

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ABSTRACT

We developed a mouse model with tongue cancer, which was useful to evaluate the induction of tumor-specific immunity. We used two kinds of squamous cell carcinoma (KLN-205 and Sq-1979) derived from mice and examined appropriate doses of each cell line for inoculation. When $2\times10^5$ KLN-205 cells were inoculated into the tongue of the mouse, 100% of tumor incidence occurred and all mice were killed by KLN-205 tumor about 23 days after tumor inoculation, and therefore, $2\times10^5$ KLN-205 cells were determined to be the optimal choice for inoculation into the tongue of the mouse. However, when $5\times10^5$ Sq-1979 cells were injected into the tongue, there were some cases of tumor regression. Our newly developed mouse tongue cancer model seems to be adequate for the assessment of standard therapy against tongue cancer and to be valuable for experiments in tumor vaccine therapy.

## INTRODUCTION

The effective treatment of squamous cell carcinoma (SCC) of the oral cavity is a worldwide health care issue[1]. Lundy *et al.*[2] verified that patients with oral cancer have impaired immune function, and demonstrated decreased delayed hypersensitivity reactivity (DTH). Immune dysfunction in patients suffering from oral cancer is of prognostic importance, and the direct correlation with disease-free survival has been established. To increase and induce tumor-specific immunity, and to reduce a tumor's propensity for invasion is of significant importance in the research and development of current medicine.

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Development of such strategies will require preclinical examination using established animal models in which effects on immune expression and invasion *in vivo* are easily quantifiable. To date, various animal models of oral cancer have been established[3~8]. However, they have not permitted careful analysis of the mechanisms of tumor-specific immunity. Although many investigations on tumor immunity have used human cancer implanted into nude mice or chemically tumorformed syngeneic cells, it is impossible to analyze the immunological function of the host for cancer in these experimental models.

We established an *in vivo* mouse model to analyze tumor-specific immunity for oral cancer, which is essential for close study of the nature of the immune response to tumor and the role of biologic modifiers.

## MATERIALS AND METHODS

*Squamous cell carcinoma cell lines and mice*

We used two squamous cell carcinoma cell lines, which were derived from DBA/2 (KLN-205) and C3H (Sq-1979) mice. These lines were obtained from Riken Cell Bank (Tsukuba, Japan). KLN-205 cells and Sq-1979 cells were maintained in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 50U/mL penicillin (all media and additives from Gibco). 100 $\mu mol$/L nonessential amino acids were also supplemented in the media of KLN-205 cells. Cultured cells were harvested, washed extensively in phosphate-buffered saline (PBS), and inoculated into DBA/2 or C3H mice.

DBA/2 and C3H mice (female, 6 weeks old) were purchased from SLC (Hamamatsu, Japan). All the mice were individually randomized, divided into 5 groups, and were ear-tagged. The number in each group was from 10 to 12.

*Cancer inoculation*

All mice were anesthetized by diethyl ether. A 40 $\mu l$ solution containing $5\times10^4$, $1\times10^5$, $2\times10^5$, $5\times10^5$ KLN-205 or Sq-1979 cells was injected into the tongue of each mouse in groups I to IV using a 100 $\mu l$ syringe and 30 gauge needle. As a control group, group V mice were injected with PBS alone. In order to compare the ecocline of the mice in different oncogenetic parts, $5\times10^5$ and $1\times10^6$ KLN-205 cell lines were inoculated subcutaneously in the right flank of DBA/2 mice, using the same method. The mice were maintained in standard animal housing up to death. All animal experiments were approved by the Animal Care and Use Committee of Yokohama City University School of Medicine.

The first study was performed on 12 mice in each experimental and control group to examine the natural course of the tumor. Body weight and tumor size were recorded every day. Tumor volume was calculated using the following formula;

Volume ($mm^3$) = $0.5\times a\times b^2$ (a: tumor's length, b: tumor's width).

*Histological examination*

Three mice were randomized and sacrificed every week after tumor inoculation. Lymph nodal, pulmonary and hepatic metastasis were identified in a blinded coded fash-

ion every week. To evaluate the histopathologic findings of tumor invasion, the submandibular lymph node, the tongue, the lung and the liver of each mouse were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Serial 3 $\mu m$ sections were prepared and stained with hematoxylin and eosin (HE). The sections were then examined by two authors simultaneously. Tumor invasion and metastasis were evaluated and recorded for each animal.

## RESULTS

*In KLN-205 cell lines groups*

Inoculated tumors were formed and grew in each experimental group, such as 8 of 12 mice in the $5\times10^4$ cells experimental group and 9 of 12 mice in the $1\times10^5$ cells experimental group. All mice formed an inoculated tumor mass in the $2\times10^5$ and $5\times10^5$ cells experimental groups when KLN-205 cell lines were inoculated into the tongue tissue of DBA/2 mice (Fig. 1). The growth rate of inoculated tumors was about 0.5 mm daily in each experimental group (Fig. 2). When the volume of tumor increased to about 138.6 $mm^3$, the mice died of their tumors. The average survival period from inoculation until death was about 23 days (Fig. 3). In addition, when the tumor volume grew over 5 $mm^3$, the body weights of the mice started to decrease gradually (Fig. 4). The mean weight of mice after oncogenesis was 22.8 g and the weight of dead mice was 12.6 g. On the other hand, the body weight of mice in the control group was 26.5 g when the mice with tongue cancer died (Fig. 5).

When $5\times10^5$ of KLN-205 cells were subcutaneously inoculated in the right flank of DBA/2 mice, 7 of 10 mice formed inoculated tumor masses, and all mice formed inoculated tumor masses in the $1\times10^6$ cells experimental group. The growth rate of abdominally

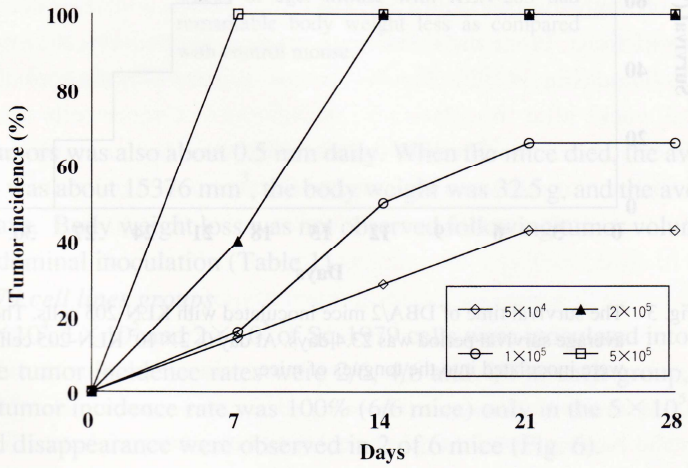


Fig. 1 Time course of each dose of KLN-205 inoculated into the tongue of DBA/2 mice. When $2\times10^5$ and $5\times10^5$ KLN-205 cells were inoculated, tumor masses were produced in all mice.

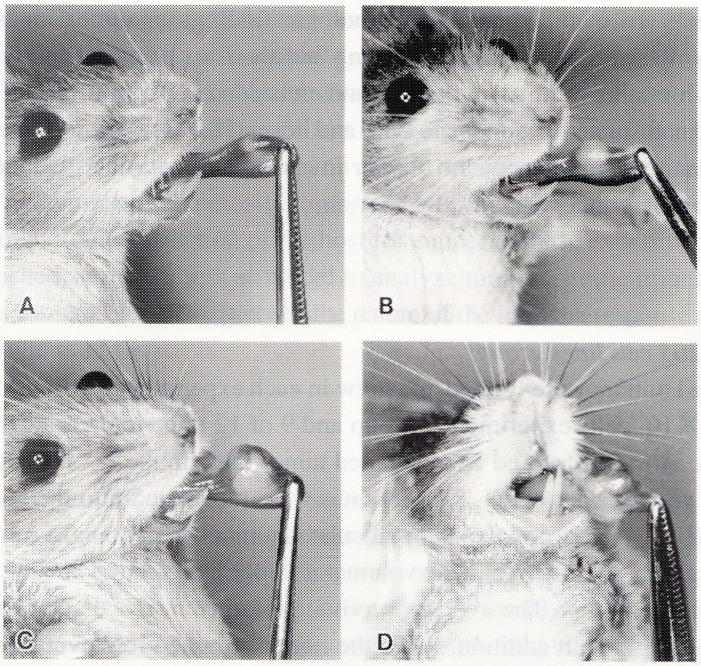


Fig. 2 KLN-205 tumor inoculated into the tongue of DBA/2 mice. A: the first week, B: the second week, C: the third week, D: the fourth week after KLN-205 inoculation.

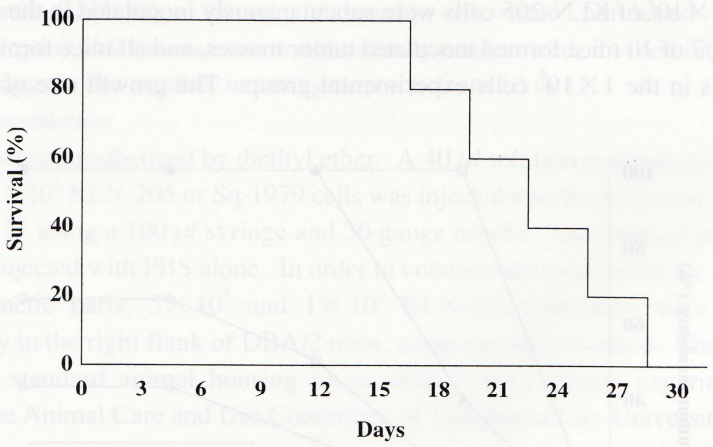


Fig. 3 The survival time of DBA/2 mice inoculated with KLN-205 cells. The average survival period was 23.4 days. At day 0, $2 \times 10^5$ KLN-205 cells were inoculated into the tongues of mice.

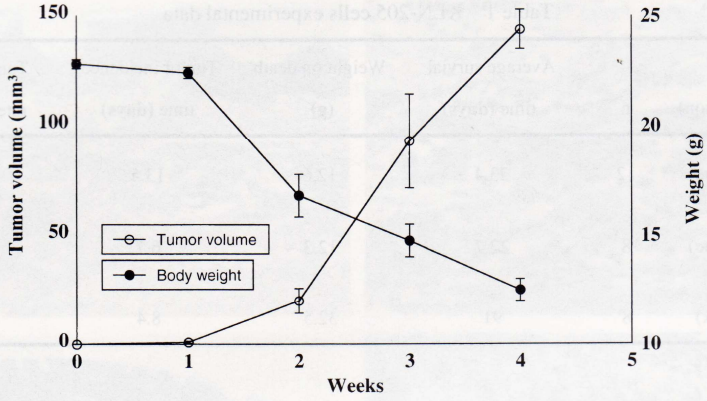


Fig. 4 Change of tongue cancer volume and body weight in DBA/2 mice after KLN-205 inoculation. When the tumor volume increased over 5 $mm^3$, body weight started to fall.

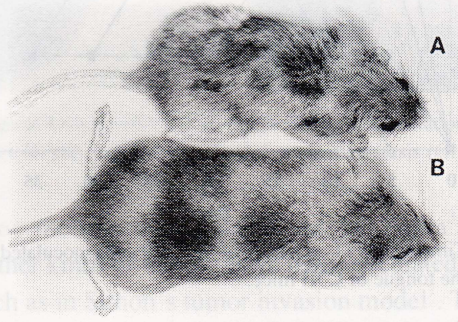


Fig. 5 Comparison of KLN-205-tumor bearing mouse (A) with control mouse (B) at 10 weeks of age. Mouse with KLN-205 had remarkable body weight less as compared with control mouse.

transplanted tumors was also about 0.5 mm daily. When the mice died, the average volume of tumor mass was about 15376 $mm^3$, the body weight was 32.5 g, and the average survival time was 91 days. Body weight loss was not observed following tumor volume increase in the case of abdominal inoculation (Table 1).

*In Sq-1979 cell lines groups*

When $5\times10^4$, $1\times10^5$ and $2\times10^5$ of Sq-1979 cells were inoculated into the tongue of C3H mice, the tumor incidence rates were 2/6, 4/6 and 4/6 in each group, respectively. Although the tumor incidence rate was 100% (6/6 mice) only in the $5\times10^5$ group, tumor regression and disappearance were observed in 2 of 6 mice (Fig. 6).

*Histological findings*

Fig.7A and B show a low-power view of the mice tongue tissue at 3 weeks after inoculating KLN-205 cells. Many cells showed nuclear division in the lingual muscle tunic.

Table 1 KLN-205 cells experimental data

| Group cell dose (region) | n | Average survial time (**days**) | Weight on death (**g**) | Tumor incidence time (**days**) | Tumor growth rate (**mm/day**) |
|---|---|---|---|---|---|
| $2\times10^5$ (tongue) | 12 | 23.4 | 12.6 | 13.5 | 0.5 |
| $5\times10^5$ (tongue) | 8 | 22.7 | 12.3 | 6.7 | 0.5 |
| $1\times10^6$ (flank) | 8 | 91 | 32.5 | 8.4 | 0.5 |

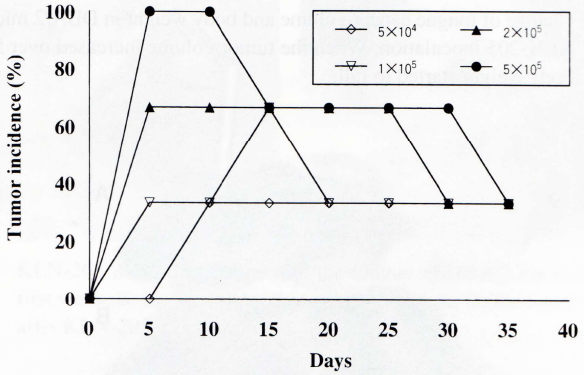


Fig. 6 Time course of each dose of Sp-1979 cells inoculated into the tongue of C3H mice.

Few infiltrating lymphocytes were observed in the muscle tunic near the mucosa. Inflammatory swelling was observed in cervical lymph nodes at the first and second weeks after tumor inoculation. From the third week, metastasis of cervical lymph nodes was microscopically found (Fig. 7C, D). Fig.7C is a low-power view of a cervical lymph node of a mouse at 3 weeks after inoculating KLN-205 cells. Cancer cells showed invasive growth. A higher-power view (Fig.7D) shows widespread nuclear division, like Fig.7B. There was no evidence of pulmonary or hepatic metastasis in the $2\times10^5$ cell experimental group (data not shown).

## DISCUSSION

In recent years, a great deal of attention has been paid to the study of the development of tumor immunity in the oral cavity[9~11]. Investigation into tumor immunity for oral cancer will require preclinical examinations using established animal models which not only have normal immune effects but also are able to provide many syngeneic tumors, so that tumor-specific immunity can be investigated. Until now, various animal models for oral cancer have been established[3~8]. One kind of such models was that of chemical carcinogenesis, for

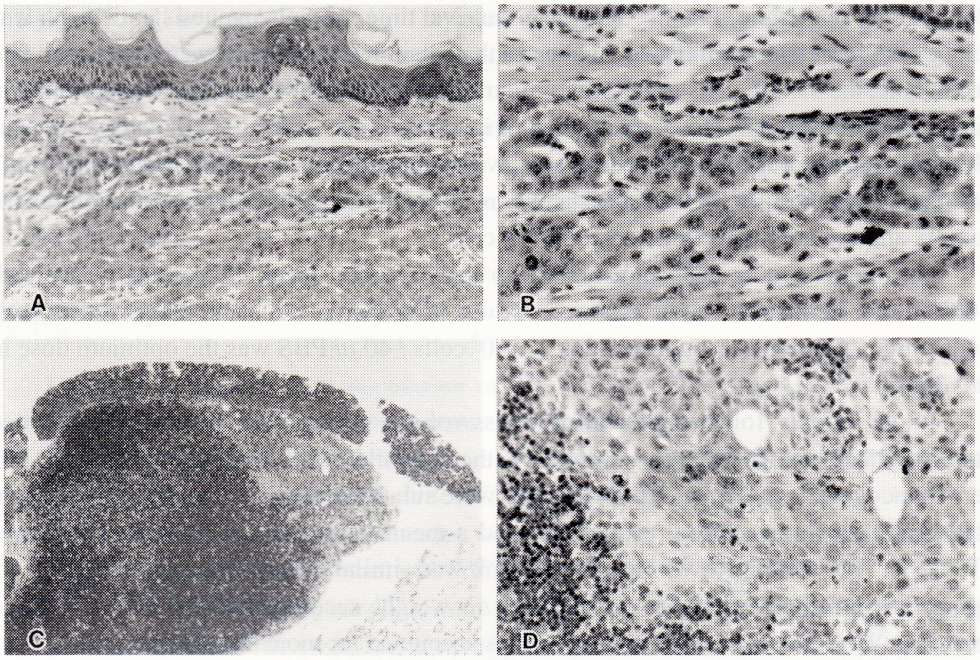


Fig. 7 Histological findings at 3 weeks after $2\times10^5$ KLN-205 cells inoculation. A, B: KLN-205 tumor in the tongue (A: HE×40, B: HE×100). C, D: Lymph node metastasis (C: HE×40, D: HE×100).

example, tongue tumor models were established by Fujita[3] using hamsters and by Hawkins[4] using rats. Other kinds of models have used inoculated tumor cells of human origin into nude mice, such as in Simon's tumor invasion model[7]. These models have played an important role in research on oral cancer, but require considerable time for oncogenesis. Furthermore, the developed tumor cells are polyclonal. Although the inoculated tumor models could use monoclonal cancer cells, the hosts must be nude mice guarantee that the host immune system will not attack the inoculated tumor cells. Therefore, these models seem unsuitable for use in the study of tumor immunity and of development of tumor vaccine.

In this study, we founded an available tongue cancer model using inbred DBA/2 mice with normal immune systems to investigate tumor immunity for oral cancer. KLN-205 cell lines, harvested from syngeneic mice, were chosen as the inoculating tumor cells. In the DBA/2 mice tongue cancer model, the oncogenetic rate was about 62% in the groups of $5\times10^4$ and $1\times10^5$ cells, so we thought that this model was not well suited for analysis of tumor immunity. The oncogenetic rate, however, was 100% in the groups of $2\times10^5$ and $5\times10^5$ cells (Fig. 1). Tumor incidence time averaged 13.5 days from tumor inoculation to oncogenesis in the $2\times10^5$ cell group. The shortest tumor incidence time was 5 days. After the transplanted tumor was produced, the tumor size gradually increased, averaging 0.5 mm every day. Following the tumor mass volume increase, the body weights of mice started to decrease (Fig. 4). When mice died of tumor growth, the average body weight was only 12.6 g (Table 1). In sharp contrast, the average body weight of mice with no tumor was

26.5 g at the same time (Fig. 5). The mean survival time from oncogenesis until death was 23.4 days (Table 1, Fig.3). Table 1 indicates that when KLN-205 cells were inoculated into the tongue, the $5\times10^5$ cells group was similar to the $2\times10^5$ cells group in average survival time and death weight. However, the tumor incidence time of the $5\times10^5$ cells group was shorter than that of the $2\times10^5$ cells group, averaging 6.7 days, with the shortest one being one day. These results showed that there was no relationship between the growth rate of tumors and the number of inoculated tumor cells.

We think the most ideal animal model to analyze tumor-specific immunity is, one with 100% tumor incidence, can be produced by use of minimum cells and longer tumor incidence time. Our results suggested that $2\times10^5$ cells / 40 $\mu l$ PBS was the optimum dose in this study.

In our models, following the tumor mass volume increase, the body weight of mice started to decrease. In order to examine whether a similar result would be obtained in different oncogenetic parts, $1\times10^6$ tumor cells were subcutaneously inoculated into the right flank of DBA/2 mice. The results indicated a mean tumor incidence time of 8.4 days (Table 1). The tumor growth rate in the flank was similar to that of tongue tumor, averaging 0.5 mm every day, but no decrease in weight accompanied the tumor volume increase. When the mice died, the tumor mass accounted for more than 1/2 of the body surface of the mice, and ulcerated carcinomas were observed. The mean survival time of the flank cancer model was longer than that of the tongue cancer model, at 91 days (Table 1). The above result showed that the tumor growth rate was similar in different parts inoculated with tumor cells but the survival time was very different.

We think that since the oral cavity and tongue of the mouse are small, tongue movement was disturbed following the tumor volume increase, and then feeding and respiratory disorders led to dystrophy. When the tumor took up a large part of the tongue and the oral cavity was filled with tumor mass (Fig. 2D), the tongues of the mice were inhibited from movement, and the mice could then no longer close their mouths, and eventually died of dystrophy. For tumor cells inoculated into the right flank of mice, the tumor growth did not influence the feeding and respiration, and the survival time of this group was longer. This result suggested that tumor growth directly affected the survival period of mice in the tongue tumor model.

On the other hand, the oncogenetic rate of the $5\times10^5$ cell group was 100% in the Sq-1979 cell model, but tumor regression was observed (Fig. 6). This phenomenon may result from the stronger antigenicity of Sq-1979 than that of KLN-205 or the poor growth of Sq-1979. Although it was necessary to try inoculations greater than $5\times10^5$ cells, it was difficult to transplant the tumor into the tongue using more than $5\times10^5$ cells from the point of view of experimental technique. Therefore we concluded that Sq-1979 cells were inadequate for our tongue tumor model.

In normal clinical conditions, the metastatic rate of lymph nodes in patients with tongue cancer is highest among patients with various kinds of oral cancer. From the third week, metastasis of cervical lymph nodes was also observed using pathological techniques (Fig.7C,D). Due to the small number of cases in this study, the metastasis rate could not be

analyzed. It will be investigated again in future using additional more cases.

Inbred mice are different from nude mice and have normal immune systems, so this tongue cancer model can be used for MHC immunity analysis. This has the advantages of not only being easy to do but also of providing many uniform animal models at the same time. Our tongue cancer model should prove very useful for the study of development and evaluation of immunotherapy, chemotherapy, and radiotherapy in the oral and maxillofacial fields in future.

## CONCLUSIONS

1. It was recognized that inoculation of $2\times10^5$ KLN-205 cells into the tongue of DBA/2 mice formed invasive tumors at 100 percent.
2. Death of mice was caused on average at 23.4 days after oncogenesis, and was due to feeding and respiratory disorders following tumor growth.
3. Metastasis of cervical lymph nodes was observed in 3 weeks after inoculation, but pulmonary or hepatic metastasis was not found.
4. The above results suggested that our tongue cancer model used in this study was an ideal experimental model for the study of tumor immunity in the oral cavity, in which a 100% tumor rate can be produced using inbred mice.

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