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Augmented Immunogenicity of Lewis Lung Carcinoma by Infection with Herpes Simplex Virus Type 2

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Abstract—In vitro, *LLT cells sustain HSV-2 replication without evidence of lysis. Simultaneously, multiplication of the cells is stimulated. These xenogenized cells were tested for their immunopotentiating capacity: three-step immunization with xenogenized viable cells conferred significantly augmented transplantation resistance to a challenge graft with* 4×10^4 *intact LLT cells. Latency periods preceding tumor formation were increased and* 15% *of the mice failed to form primary tumors. Metastasis was likewise decreased and* 25% *of the mice had healthy lungs. Immunopotentiation, however, did not suffice to significantly protect against a challenge with* 6×10^4 *intact cells. The presence of virus-specific neoantigens on HSV-2-infected viable cells was demonstrated by the progressively increasing number of rejections of* 4×10^4 *xenogenized cells during the successive immunization steps. Immunization with non-viable LLT cells did not augment resistance to challenge.*

INTRODUCTION

THE PHENOMENON of augmented tumor-specific transplantation antigen (TSTA) activity resulting from antigenic modification of tumor cells by means of virus infection has been reported by a number of investigators. There are two methods available: (a) the augmented antigenicity of tumor cell homogenates or membrane extracts after infection with cytopathic viruses such as influenza virus [1–4], vesicular stomatitis virus [5–7], Newcastle disease virus [8] and vaccinia virus [9] (for review see [10]), and (b) the augmented TSTA activity of intact tumor cells capable of proliferation *in vivo* after persistent infection with partially cytopathic or noncytopathic viruses such as Sendai virus [11], measles virus [12], lymphocytic choriomeningitis virus [13], various oncornaviruses [14–16] and others. For the latter method Kobayashi had coined the term of 'xenogenization' (for review see [17]).

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Lewis lung carcinoma was considered a suitable choice for immunization experiments because (a) it elicits no immunologic response whatsoever [18, 19], and (b) this tumor would allow us to determine whether immunization with virus-infected cells could influence metastasis. Griffith *et al.* [20] have shown previously that the life span of mice challenged with intact LLT cells was increased due to immunization with tumor cell membranes after infection with influenza virus.

Herpes simplex virus type 2 (HSV-2) was selected when it had become apparent that it proliferated in LLT cells *in vitro* without causing cytopathic damage, a prerequisite for xenogenization. In fact, it has been ascertained that viable xenogenized cells were better immunopotentiators than were either non-viable cells or cell membranes [21]. It was found, furthermore, that the infection of LLT cells with HSV-2 stimulated their proliferation above control cultures—another factor which might enhance the antigenic stimulus.

In fact, immunization with such cells confer-

red a high degree of transplantation resistance to the xenogenized cells themselves and resistance was clearly increased with each immunization step. When the animals were challenged with intact LLT cells, primary tumor formation was significantly slowed and tumor incidence was reduced by 15%. The growth of metastases was delayed and 25% of the immunized mice had intact lungs. In contrast, immunization with non-viable cells conferred no resistance.

MATERIALS AND METHODS

Tumor

Lewis lung carcinoma (LLT) is a malignant tumor which metastasizes in the lungs and never regresses spontaneously. It originated in the lung of a C57BL/6 mouse [22] and was maintained in B6/D2F1 mice by serial transplantation of primary tumors every 14–16 days.

Virus

HSV-2, strain MS, was propagated in the permanent cell line CV1 (green monkey kidney). Virus and cells had been stored in liquid nitrogen at their second passage after arrival from the American Type Culture Collection. Titration of the virus was performed on CV1 cells in microtest plates (Flacon 3034, Falcon Plastics, Oxnard, CA) and contained 3.5×10^7 ID_{50}/ml.

Cell cultures and infection with HSV-2

CV1 cells were propagated in Eagle's MEM with Earle's salts supplemented with a two-fold standard concentration of amino acids. LLT cells were cultured in RPMI 1640. Both culture media were enriched with 10% heat-inactivated fetal calf serum (FCS). For infection, 10^4 cells were planted in 75-cm^2 plastic tissue culture flasks (Falcon Plastics, Oxnard, CA). When the cells were 70–80% confluent about 3 days later, the medium was decanted. CV1 cells were infected at approx. 1 m.o.i., LLT cells at approx. 2 m.o.i. Following adsorption for 90 min at 37°C the cells were washed twice with prewarmed Hank's balanced salt solution, and 15 ml medium were added to the monolayers which were then incubated further. CV1 cells which showed extensive cytopathology were harvested 24 hr post-infection (p.i.). LLT cells, however, were incubated for an additional 48 hr since no change in morphology was detected.

Mice

B6/D2F1 mice were bred at the Institute for Cancer Research, Vienna, Austria. All experiments were conducted with mice about 3 months old, matched for age and sex.

Evaluation of primary tumors and metastases

Following implantation of neoplastic cells into the thigh muscle (i.m.) primary tumors were measured by callipers in two directions and their volume was calculated according to the formula $V = (0.4)\ (ab^2)$, where $a > b$ [23]. Those reaching 0.025 cm^3 after substraction of the measurements obtained with the opposite intact thigh were considered to be positive.

Metastases were made visible according to the method of Wexler [24] and were counted under a low-power dissecting microscope.

Preparation of LLT cell suspensions and xenogenization

Several primary tumors trimmed of muscle and necrotic tissue were minced. Single cell suspensions were obtained by digestion with 0.25% bacto-trypsin (Difco Labs, Inc.) and subsequent filtration through a 400 mesh stainless steel gauze. They were stored in small aliquots in liquid nitrogen. Before use the cells were rapidly thawed and gently shaken to re-suspend. They were filtered through a double layer of cotton gauze and the number of viable cells was assessed by trypan blue exclusion technique.

For xenogenization, suspensions of viable LLT cells were infected with HSV-2 at approx. 8.5 m.o.i. and incubated for 2 hr at 37°C under frequent shaking. Finally, the suspensions were washed to eliminate non-adsorbed virus particles and adjusted to contain 4×10^5 cells/ml.

Experimental design of the in vivo *experiments*

Figure 1 represents the immunization, challenge and control procedures. Group A consisted of 50 mice which were immunized with xenogenized cells. Group B comprised 20 animals which were treated with cells made non-viable by repeated freezing. The immunization procedure for groups A and B consisted of one injection of 4×10^4 cells into a thigh muscle and two subcutaneous (s.c.) inoculations of the respective single cell suspensions near one of the inguinal folds after 17 and 12 days respectively. Twelve days later both groups were challenged into the contralateral thigh muscle with 4×10^4 and 6×10^4 intact cells respectively. Primary tumor development was monitored thereafter and 15 days later the mice were killed and the number and size of metastases was determined.

Group C comprised 10 mice which were in-

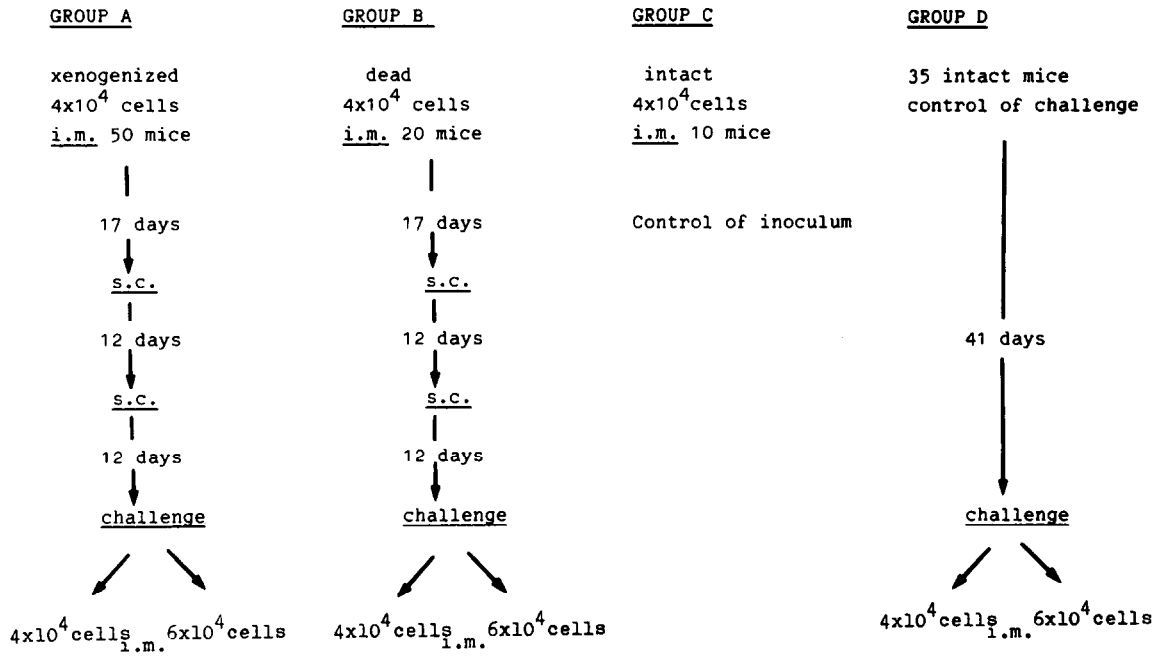

Fig. 1. Schematic presentation of immunization, challenge and control procedures.

oculated i.m. with 4×10^4 sham-infected cells simultaneously with the first i.m. immunization step and served as a control for the viability and oncogenic potential of the cell suspensions. Group D, consisting of 35 mice of equal age and sex as those of the previous groups, was left intact. The mice were injected with the challenge material simultaneously with the test mice. They served as controls for the challenge material and provided the baseline values for tumor incidence and growth after challenge.

Statistical significance of the results was evaluated according to a preliminary *F* test and Student's *t*-test.

RESULTS

Infection of LLT cells in vitro *with HSV-2*

Eight flasks were seeded with 10^4 cultured LLT cells of which, 3 days later, four were infected with HSV-2 and the remaining flasks were sham treated. Simultaneously, a flask containing a monolayer of CV1 cells was infected with the same virus suspension. The latter, after 24 hr incubation, showed extensive cytopathic alterations. The virus content was 2×10^7 $TCID_{50}$/ml. In contrast, no cytopathic effect could be detected in the infected LLT monolayers, even though the incubation period had been extended to 3 days. Furthermore, the infected cultures, compared with the controls, appeared more densely populated and more anchorage-dependent (Fig. 2). The cells were then detached by vigorous shaking and their number in each flask was determined. Thus the infected flasks had an average content of about 10^6 viable cells against only 4×10^5 viable cells in the control cultures. Furthermore, replication of the virus had taken place: an approx. 50-fold increase above the input amount was ascertained and the average titre reached 2.3×10^6 $TCID_{50}$/ml.

Thus, *in vitro*, the infection of LLT cells with HSV-2 is productive but has no cytolytic effect. In addition, the infection stimulates cell multiplication.

Immunization

Twenty mice of group B which had received non-viable cells remained free of tumors throughout all immunization procedures. Of the 50 mice of group A which had been treated with viable xenogenized cells, only 13 (26%) had neoplasias at the site of the first immunization step (i.m.) against 100% of 10 control mice of group C, which had been inoculated with the same number of sham-infected cells. In addition, in the test group the first tumor appeared 12 days and the last tumor 20 days after inoculation, in contrast to the control group, which produced all tumors between the 7th and the 11th days.

Since it can be presumed that, in analogy to the situation encountered *in vitro*, LLT cells infected with HSV-2 remain fully capable of proliferation once they are introduced into liv-

ing organisms, it is reasonable to suppose that 74% of the mice in group A had successfully rejected the xenogenized inoculum.

After the tumor-bearing animals had been discarded, the remaining 37 mice were subjected to further immunization steps: only 6 (16.6%) developed neoplasias following both s.c. injections with 4×10^4 xenogenized cells. Their formation was preceded by long latency periods since none had been detected when the animals were challenged 12 days after the second s.c. injection.

Thus the gradual decrease of tumor incidence in group A in response to three successive immunization steps is suggestive of an increasing resistance to the xenogenized cells as such.

Challenge with intact LLT cells

The challenge material, consisting of 4×10^4 and 6×10^4 cells respectively, was injected into the contralateral thigh muscle. Of 37 tumor-free mice left in group A, 27 received the lower and 10 the higher cell dose. Simultaneously, 25 control mice (group D) were inoculated with the lower and 10 with the higher dose. As shown in Fig. 3, the results demonstrated that immunization with xenogenized cells conferred significant resistance to the challenge with 4×10^4 intact LLT cells. Thus, in group A the latency period was increased: on day 7 p.i. only about 10% had tumors, against 70% in the control group D. By day 11 p.i. tumor incidence in group A had not yet reached 50%, in contrast to the controls where tumor incidence was approaching 100%. Finally, 4 (15%) of the immunized mice remained free of primary tumors against 0% in the control group.

Determination of growth rates of primary tumors provided an additional parameter for assessing transplantation resistance. Thus the mean tumor size per mouse in groups B and D increased rapidly, whereas it was significantly slower in group A. As shown in Fig. 4, in the latter group the neoplasias nowhere reached half the dimensions of the controls.

On the other hand, it became apparent that immunization with non-viable, non-xenogenized cells (group B) conferred no significant transplantation resistance.

The examination of the lungs 15 days after challenge revealed that immunization with HSV-2-infected viable cells conferred a significant protection against metastasis: in group A their number and size was significantly inferior to those in the control group and 7 out of 27 mice (approx. 25%) had completely healthy lungs, compared with none in the latter group. Conversely, immunization with non-viable cells did not protect the hosts against metastasis (see Fig. 5).

Alternatively, resistance to challenge with 6×10^4 cells was negligible and statistically insignificant. Similar inconclusive results were obtained when metastases were examined (results not shown).

DISCUSSION AND CONCLUSIONS

The infection of LLT cells with HSV-2 takes an unusual course in that they sustain proliferation of the virus without being subjected to lysis. This property should be of interest for routine diagnostic purposes as well as for HSV research. Another exceptional feature is the simultaneously enhanced multiplication of the cells, a phenomenon possibly related to the growth-stimulating factor which was found to accumulate in the medium of lytically infected cell cultures [25, 26].

These properties of LLT cells predestined them for xenogenization assays since according to Beverley *et al.* [8], these features are essential for obtaining augmented immunogenicity.

A further requirement for augmentation of TSTA by the xenogenization method to take place is the induction of virus-specific cell-surface antigens [27]. In fact, such membrane-bound neoantigens are synthesized by HSV and are, possibly, identical to glycoproteins specified for by the virus [28, 29]. These neoantigens were undoubtedly present on xenogenized LLT cells and were best demonstrated by the failure to produce tumors in 74% of the mice which had been inoculated i.m. with such cells, in contrast to 0% of the control mice, which had received the same number of intact cells. Furthermore, the capacity for rejection of xenogenized cells increased progressively with each immunization step. In addition, it is probable (although it cannot be proved) that *in vivo*, similar to the *in vitro* results, the infection of LLT cells causes an enhancement of their multiplication rate.

Transplantation resistance to challenge with 4×10^4 intact LLT cells was likewise obtained, though to a lesser extent: the latency period for primary tumor formation was significantly increased, growth was slower and 15% of the mice failed to develop neoplasias, compared with 0% of the control animals. Lung metastasis was also reduced, and whilst none of the control mice had healthy lungs at the time they were killed, 25% of the animals of group A were free of metastatic foci. The latter result is of special interest since the role of host immunity in metastasis is not yet well defined [30].

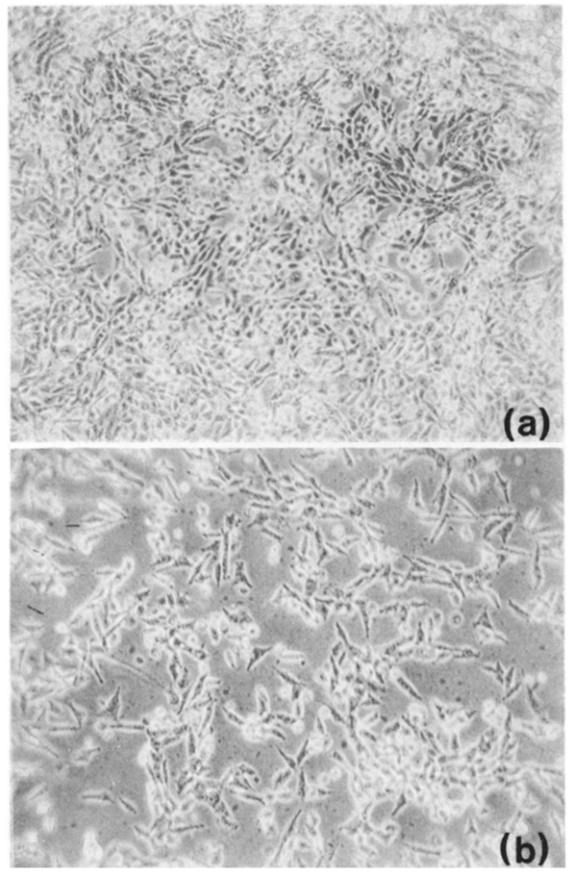

Fig. 2. Phase contrast micrographs of HSV-2-infected LLT cell cultures 72 hr *p.i.* (a) *Infected cells* (*magnification* 85/1); (b) *sham-treated cells* (*magnification* 85/1).

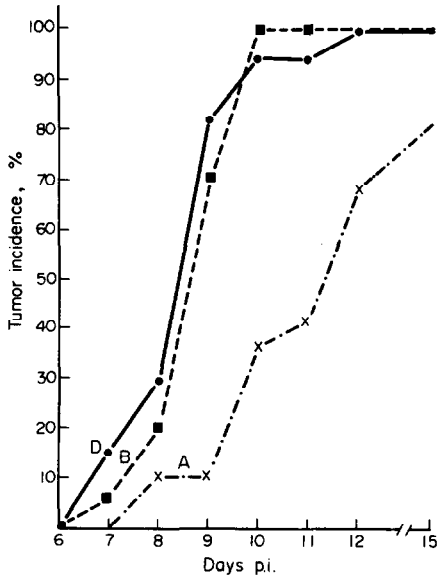

Fig. 3. Primary tumor incidence in mice immunized with xenogenized cells (group A), with non-viable cells (group B) and in intact mice (group D) following challenge with 4×10^4 intact LLT cells. Of the mice in group A, 15% remained free of tumors.

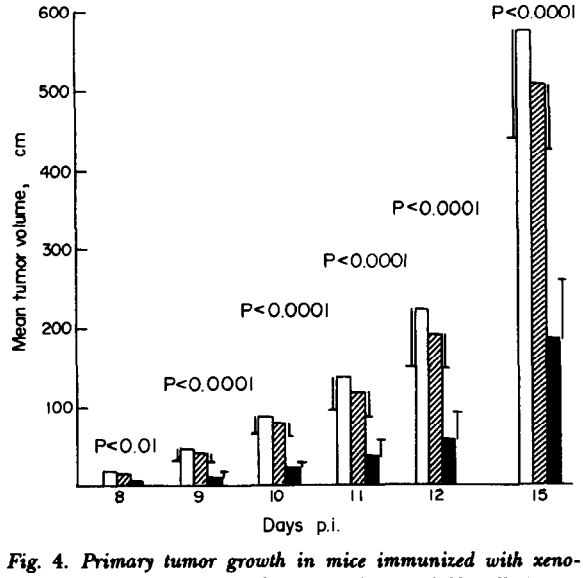

Fig. 4. Primary tumor growth in mice immunized with xenogenized cells (group A): dark bars; with non-viable cells (group B): hatched bars; and in intact mice (group D): white bars; following challenge with 4×10^4 intact LLT cells. Results of Student's t-test refer to statistical differences between group A on the one hand and groups B and D on the other hand, which do not differ statistically. Calculations include tumor-free animals.

In contrast, immunization with xenogenized cells proved to be almost completely ineffective against a challenge with 6×10^4 LLT cells. This rather elevated dose obviously allowed many cells to escape immunosurveillance. In addition, the rapid growth rate of Lewis lung carcinoma must be taken into consideration; in fact, the latency period in control mice at this dose averaged 5 days. It is therefore conceivable that cells had reached too great a number too fast to permit their efficient inactivation.

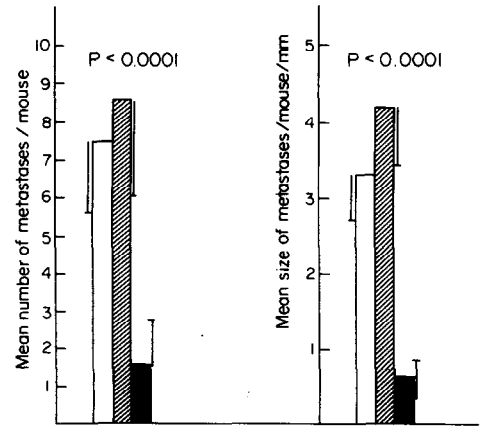

Fig. 5. Size and number of metastases in the lungs of mice immunized with xenogenized cells (group A): dark bars; with non-viable cells (group B): hatched bars; and in intact cells (group D): white bars; 15 days after challenge with 4×10^4 intact LLT cells. Results of Student's t-test refer to statistical differences between group A on the one hand and groups B and D on the other hand, which do not differ statistically.

Lower challenging doses would undoubtedly have rendered the system more sensitive. However, according to preliminary titration assays, in our hands dosages below those adopted by us tended to increase variations, thus nullifying the eventual advantages.

Finally, it should be noted that the infection of the host with HSV can modulate the growth of LLT: thus the s.c. virus infection in the vicinity of an intramuscular tumor cell inoculum significantly enhances tumor growth, whereas a similar contralateral virus infection has no consequences. This phenomenon is discussed elsewhere [31]. In contrast, as shown above, the infection of the cells prior to their implantation into mice inhibits tumor formation.

In conclusion, infection with HSV-2 of LLT cells increases their TSTA activity which, according to other authors, is ineffectual in the intact cells. Immunization with such xenogenized cells protects the hosts very efficiently against HSV-infected LLT cells. Complete rejection of intact tumor cells was successful in 15% of the animals only, but on the other hand, the development of primary tumors and of metastases was significantly slower. In contrast, immunization with non-viable cells did not augment transplantation resistance.

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