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Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. II. Establishment of specific systemic anti-tumor immunity

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The successful application of a non-oncogenic virus, Newcastle disease virus (NDV), which can be used to modify a highly metastatic tumor to become more immunogenic is reported. Such NDV modified tumor cells were found to be effective as tumor vaccine for anti-metastatic therapy in combination with surgical removal of the primary tumor. The protection in the animals seen after this treatment is paralleled by an establishment of specific systemic anti-tumor immunity. This protective immunity depended on recognition of a distinct tumor antigen. The therapy protocol also worked in animals bearing the plastic adhesive variant ESb-MP. It did not work, however, when using an immune escape variant not expressing a specific tumor antigen.

Introduction

We have recently reported an effective anti-metastatic therapy protocol in a mouse tumor model combining surgery with postoperative immunotherapy using virus-modified autologous tumor cells [9]. No therapeutic effect was observed when we used for immunotherapy the non-modified autologous tumor ESb which is only weakly immunogenic and highly metastatic [14]. The rationale for using virus-modified tumor cells for vaccination was that we intended to activate and direct such immune responses towards tumor cells which have been successfully selected through evolution for protection against infectious viral diseases. The viral modification of the tumor cells was achieved by infecting the tumors with an avirulent strain of Newcastle disease virus (NDV), thereby xenogenizing the tumor cell and increasing its immunogenicity and antigenicity [9]. NDV has been selected also because it is a very good interferon activator [10], has very low pathogenicity and neurotropism in humans and has been used already as an anti-neoplastic agent [5–7].

In a previous report [9] parameters for optimal therapeutic effects were described. These parameters were the time of operation of the primary tumor and the dose of virus admixed to a standard dose of irradiated tumor cells. There was a low dose optimum of NDV at about 160 hemagglutinating units per 25 million tumor cells.

In this report we substantiate these findings and show the effects of this therapy approach when applied to several well-defined variants of the ESb tumor line. The ESb-MP is a plastic-adhesive variant [8] which has reduced malignancy *in vivo* but

expresses a similar tumor-associated transplantation antigen (TATA) as ESb cells. Another variant is an immune escape variant from ESb which does not express this TATA to be recognizable by cytotoxic T lymphocytes [3]. We will show with this tumor variant that postoperative immunotherapy with virally modified autologous tumor cells depends on the recognition of a specific tumor antigen.

In the second part the analysis of the protective immunity induced in operated animals by using virus-modified tumor cells is reported. It is demonstrated that animals which survive due to this therapy protocol developed long-lasting systemic anti-tumor immunity. They resisted challenge inocula of life autologous tumor cells without virus. The specificity of the protective immunity will be demonstrated.

Materials and methods

Animals

Female DBA/2 mice, 6–12 weeks old, were obtained from Charles River WIGA, Sulzfeld (F.R.G.).

Tumor cell lines

ESb is a spontaneous high metastatic variant of the chemically induced T cell lymphoma L5178YE (Eb) [14]. Our standard line ESb 289 is characterized by a tumor-associated transplantation antigen (TATA) which can induce weak protective immunity and cytotoxic T cells *in vitro* [4, 15]. ESb 816 variant cells represent immune escape variants which are considered as $TATA^-$ variants which cannot be recognized by cytotoxic T lymphocytes [3]. ESb-MP cells are a plastic adhesive variant of ESb 289 cells which differ in overall malignancy *in vivo* [8]. MDAY-D2 tumor cells [11] were obtained from Dr R. Kerbel (Toronto, Canada) and have been characterized in his group as a high metastatic tumor line expressing a distinct tumor antigen. SL2 is a spontaneous T cell lymphoma of DBA/2 origin [18]. All tumor lines were grown in RPMI 1640 medium (Gibco Ltd, Paisley, U.K.) supplemented with fetal calf serum and 5×10^{-5} M 2-mercaptoethanol.

Newcastle disease virus

A stock of NDV of the avirulent strain Ulster was kindly provided by Dr Peter J. Russell (Royal Veterinary College, London, U.K.) [13]. Virus propagation in embryonated chicken eggs and purification were done as described previously [9]. Virions were finally suspended in phosphate-buffered saline to give a standard batch with a hemagglutination titer of 1 : 2000. The virus was stored prior to use at −70°C.

Postoperative immunotherapy protocol

Details of this protocol have been described [9]. Briefly, animals were inoculated intradermally in the flank region with 5×10^4 ascites-grown tumor cells. The primary tumor was removed surgically under anesthesia when it was 5 to 7 mm diameter. Directly after surgery, animals received one dose of $2{\cdot}5 \times 10^7$ tumor cells previously irradiated with 10 000 rad and suspended in 1 ml of live NDV containing 160 hemagglutinating units. This was distributed s.c. and i.m. at four different sites surrounding the operation wound.

Results

Postoperative immunotherapy with virus-modified tumor cells: therapeutic effects in animals with metastases of ESb or ESb-MP cells

We have reported before [8] that we could isolate from the highly malignant metastatic tumor line ESb a plastic adhesive variant which has lower malignancy *in vivo*. We have recently characterized in detail one particular plastic adhesive variant, ESb-MP, which has a lower malignancy *in vivo* but can still metastasize to internal organs. In figure 1 (*a*), (*b*), we have compared the results of an immunotherapy experiment in which we used animals which carried either the ESb tumor or the ESb-MP tumor for therapy with the combination of surgical removal of the primary tumor followed by immunization with virus-modified tumor cells. In both groups the primary tumors were removed when they had reached a size of about 5 to 7 mm diameters. The figures show the mortality curves of groups of animals (10/group) which were either not treated (groups I and II as control groups), operated on only (groups III or IV) or operated on and immunized with NDV-modified ESb tumor cells (groups V and VI). In figure 1 (*a*) it can be seen that in group V more than 50 per cent of the animals were long-term survivors while in the other groups the majority of animals were dead after about one month. These results which are statistically significant confirm our data reported previously [9].

Figure 1 (*b*) illustrates the results obtained in animals bearing the ESb-MP tumor. The effect of postoperative immunotherapy with NDV modified ESb cells was less pronounced but there were still more than 30 per cent of the animals surviving for longer than two months, while the operation of the primary tumor alone (group IV) did not significantly change the mortality curve of the tumor-bearing animals. In the ESb-MP tumor model mestastases in internal organs develop later, are more of the focal type and the animals die later from this tumor and its metastases. The fact that we can also obtain therapeutic effects with this tumor shows that the system might have broader applicability.

Postoperative immunotherapy with virus-modified tumor cells: comparison of $TATA^+$ and $TATA^-$ ESb tumor variants

Figure 2 illustrates the results of an experiment in which groups of animals were inoculated either with the standard $TATA^+$ tumor line ESb 289 or with the $TATA^-$ variant ESb 816. Animals in groups I and II were inoculated with 5×10^4 ESb 289 tumor cells intradermally. One group (I) was not further treated, the other (II) was treated as before by removal of the tumor and postoperative immunization with NDV-modified ESb tumor cells. The therapeutic effect can be seen by comparison of groups I and II, although some animals in group II had already died before the treatment.

Quite different results were obtained when animals were inoculated with an immune escape variant of ESb (ESb 816 tumor cells) which are $TATA^-$ variants [3]. These tumor lines were shown before to be resistant to lysis by ESb-specific cytotoxic T lymphocytes (CTL). They still expressed the $H\text{-}2K^d$ molecule which functions as a restricting element for the CTLs. Three groups of animals were inoculated with 5×10^4 tumor cells intradermally. When the tumor had reached a size of 5–7 mm in diameter, it was operated on (groups IV and V) and treated with NDV-modified ESb $TATA^+$ tumor cells (group IV) or with NDV-modified $TATA^-$ variants (group V). The mortality curves of all three groups (groups III–V)

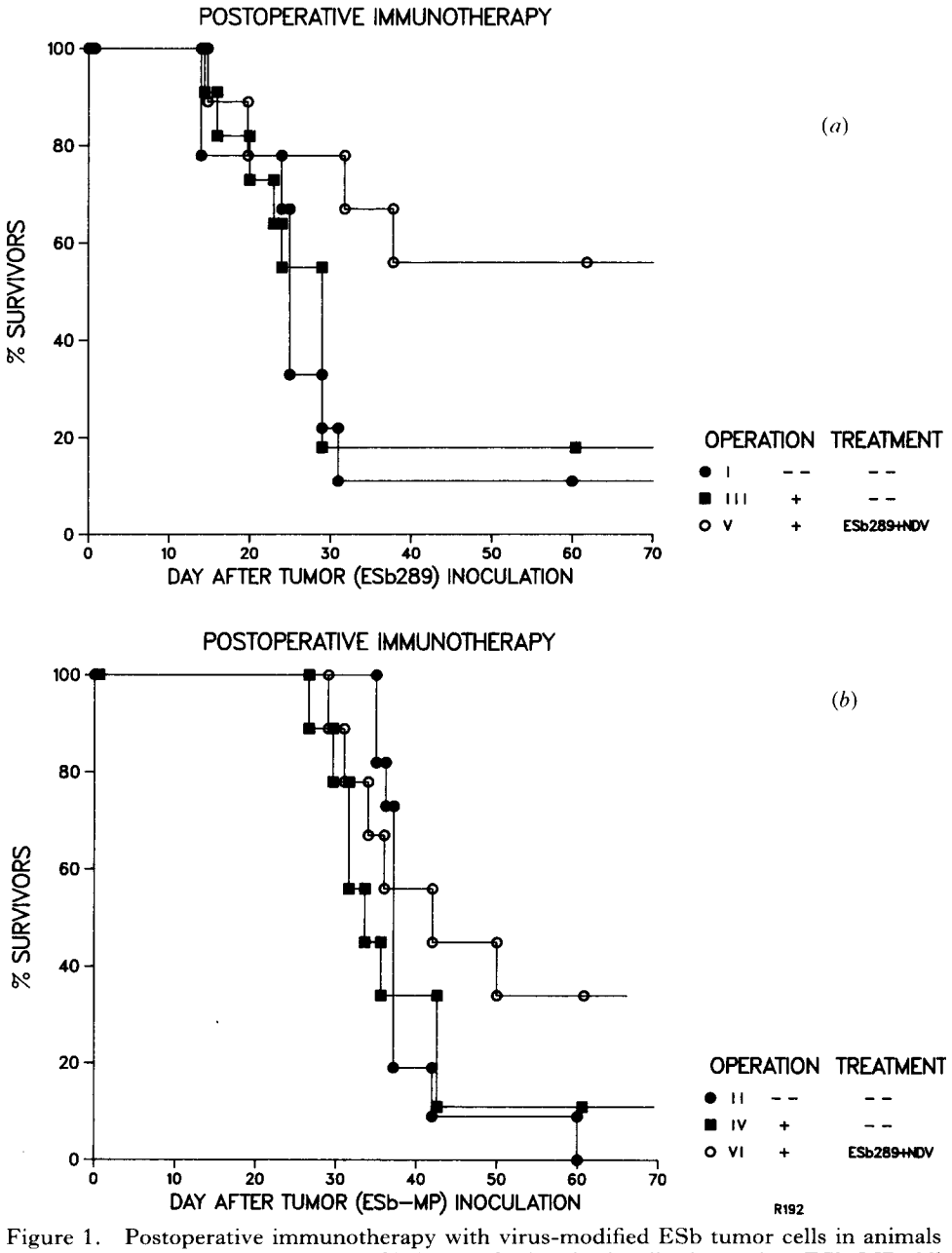

Figure 1. Postoperative immunotherapy with virus-modified ESb tumor cells in animals bearing either (*a*) the tumor ESb 289 or (*b*) the plastic adhesive variant ESb-MP. All animals were inoculated on day 0 with 5×10^4 tumor cells intradermally. The local tumors remained (groups I and II) or were removed (all other groups) when they had reached 5–7 mm in diameter. This was (*a*) at day 8 or (*b*) at day 14. Groups V and VI were immunized after the operation with 2×10^7, 10000 rad irradiated ESb 289 ($TATA^+$) tumor cells which had been incubated with 100 hemagglutinating units of NDV Ulster strain. No therapeutic effect was seen by surgery alone (groups III and IV), but protection was obtained after specific immunotherapy with ESb–NDV (groups V and VI). In both experiments (*a* and *b*) the therapy groups were significantly different from the controls. (Groups V to I: $P=0{\cdot}036$, log rank test; VI to II: $P=0{\cdot}014$, acceleration test.)

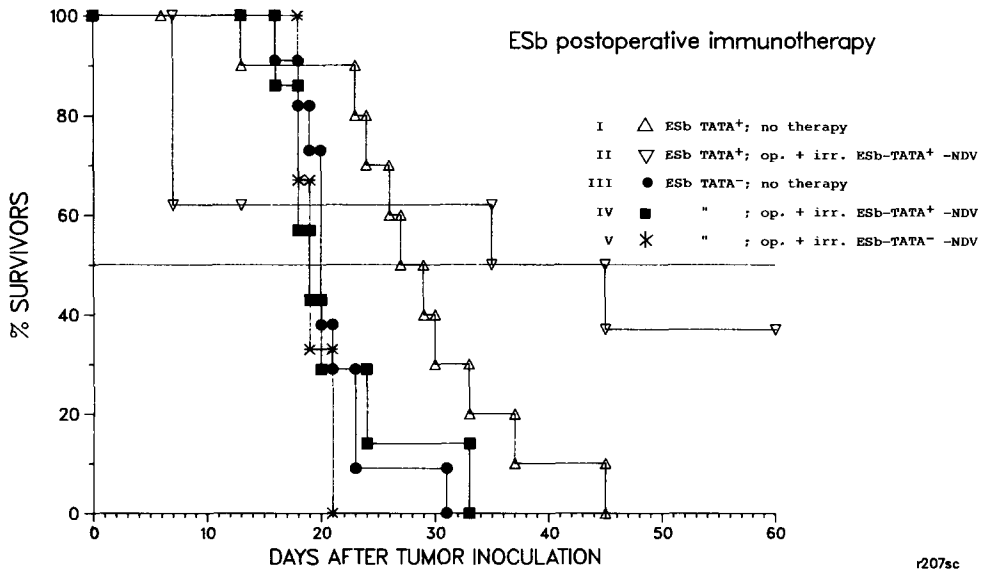

Figure 2. Importance of recognition of a tumor-associated transplantation antigen (TATA) for the therapeutic effect of immunization with virus-modified tumor cells. Animals were inoculated with 5×10^4 ESb 289 ($TATA^+$) tumor cells intradermally (groups I and II) or with 5×10^4 ESb 816 ($TATA^-$) variants (groups III–V). The animals were either not treated (groups I and III) or operated on when the primary tumor had reached a size of 5–7 mm in diameter and immunized with NDV-modified ESb 289 tumor cells (groups II and IV) or with NDV-modified ESb 816 ($TATA^-$) variant cells (group V). In none of the groups inoculated with $TATA^-$ variant tumor cells was there a therapeutic effect in comparison with the untreated group. In contrast, a therapeutic effect was seen when using the $TATA^+$ standard line (group II). Group II was significantly different from group I ($P = 0{\cdot}02$, acceleration test).

were very similar and the 50 per cent survival was not significantly different. These negative findings with $TATA^-$ cells thus point towards the importance of the expression of a tumor antigen for specific immune recognition and postoperative immunotherapy using NDV-modified tumor cells.

Specificity of protective immunity in ESb tumor-bearing animals which have been successfully treated postoperatively by NDV-modified ESb tumor cells

ESb tumor-bearing animals which had been cured by the above postoperative immunotherapy protocol were challenged either with live autologous ESb tumor cells (without NDV) or with live SL2 tumor cells. SL2 is another T cell lymphoma of DBA/2 mice which does not crossreact with ESb in immunization/protection assays and at the level of CTL lysis [4]. While SL2 tumor cells grew in all animals, ESb tumor cells did not grow (figure 3). The animals had thus developed tumor-specific immunity which was sufficient to protect them against local tumor growth (figure 3), outgrowth of metastases and death (figure 4).

Tumor specificity was also demonstrated at the level of immunity induction. This is illustrated in figure 5. Groups of DBA/2 mice were inoculated either with ESb tumor cells or with MDAY-D2 tumor cells, another high metastatic tumor of DBA/2 mice [11]. Two weeks later the animals were subjected to operation of the

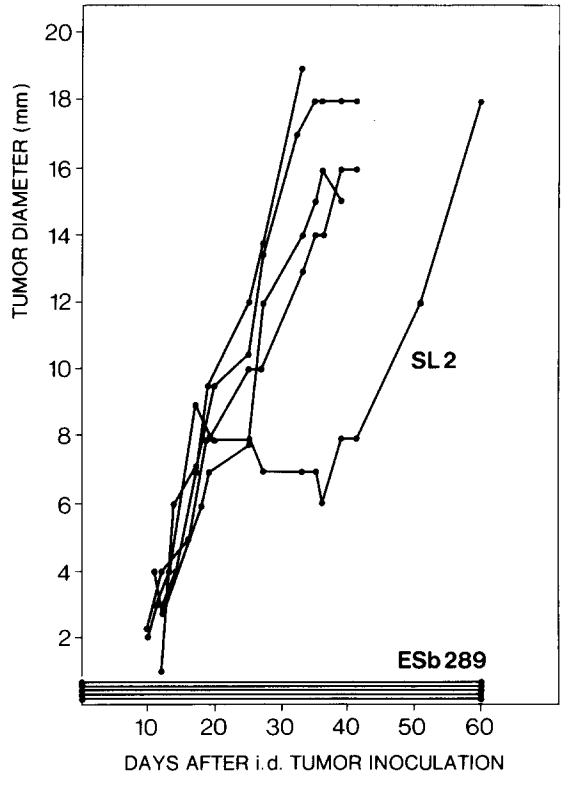

Figure 3. Specificity of the protective immunity *in vivo*. ESb tumor-bearing animals which had survived due to the combined treatment with surgery and postoperative immunization with NDV-modified ESb tumor cells were challenged after 2 months with 10^5 cells of two different DBA/2 lymphoma lines and the local tumor growth determined in individual animals. While ten animals were investigated per group only five representative growth curves are represented. While SL2 tumor cells grew out in every animal inoculated, ESb tumor cells did not grow out, thus indicating the establishment of systemic anti-ESb tumor immunity. When the two tumor lines were inoculated into normal non-immune animals, both grew out and killed the majority of the recipients (figure 4).

primary tumor and postoperative treatment with autologous tumor cells and NDV. Fifty per cent or more of such treated animals survived. After about four months animals of both groups were challenged with live ESb tumor cells. Only those which had previously been exposed to ESb tumor cells survived the challenge. It can thus be concluded that successfully treated animals had developed a long-term status of tumor-specific immunity.

Discussion

Here we extend our findings relating to a successful protocol for the therapy of micrometastases using as model the very aggressive mouse tumor variant ESb [9, 14]. The protocol consisted of the combination of surgery with postoperative immunotherapy using virus-modified inactivated tumor cells. Surgical removal of

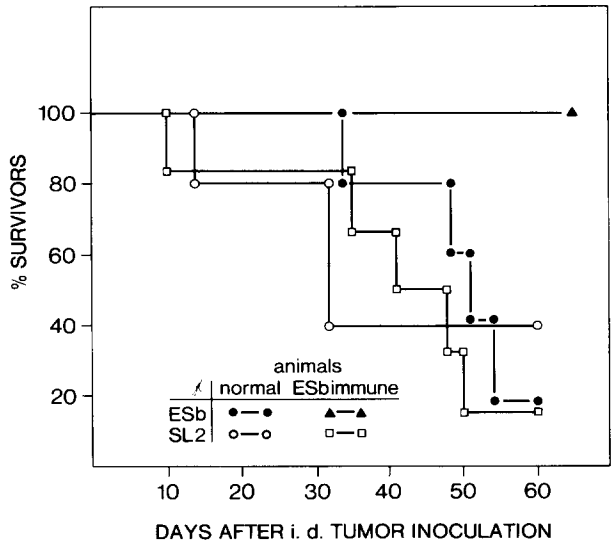

Figure 4. Survival curves of animals from the experiment illustrated in figure 3. Normal DBA/2 animals as controls or ESb immune animals which were derived from the therapy experiment (figure 3) were challenged with live ESb or SL2 tumor cells intradermally. While normal animals died from both tumor lines the ESb immune animals died only when receiving the SL2 tumor cells and not when receiving the ESb tumor cells.

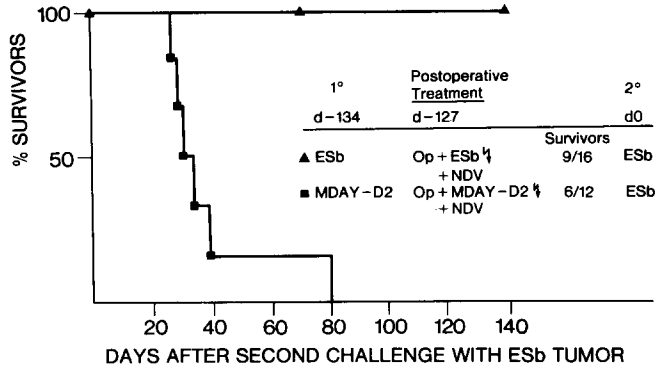

Figure 5. Specificity of protective immunity in the therapy model at the level of induction of anti-tumor immunity. Groups of DBA/2 mice were inoculated with 10^5 ESb tumor cells or MDAY-D2 tumor cells at day −134. Seven days later (day −127) the locally growing tumors were surgically removed and the animals immunized with NDV-modified ESb or NDV-modified MDAY-D2 tumor cells which had been irradiated before with 10 000 rad. After about 4 months there were still about 50 per cent of animals in both groups which had survived this treatment. These were challenged on day 0 with 10^5 ESb tumor cells subcutaneously. It can be seen that only those animals which had been pre-exposed to the ESb tumor survived while those which had been pre-exposed to the MDAY-D2 tumor died from the ESb tumor cell inoculation.

the intradermally transplanted ESb tumor without immunotherapy had no therapeutic effect and all animals died from metastases. Postoperative immunotherapy with inactivated autologous tumor cells alone was also ineffective.

For tumor cell modification it was decided to use a non-hazardous virus which has potential clinical application [5–7]. A non-lytic strain was selected because it has been reported to be more effective in increasing tumor cell immunogenicity than lytic viruses [12]. The Ulster strain of NDV quickly absorbs to the cell membrane, infects the tumor cells and as a paramyxovirus buds at the cell surface and expresses viral proteins which can then be detected with monoclonal antibodies [9]. Another advantage of this virus is its strong potency to induce interferon [10] and thus to stimulate various natural and specific immune effector mechanisms [16]. While in the previous report we presented parameters for optimal therapeutic effects using the ESb tumor system we now report on an extension of these findings to other tumor variants. A protective effect upon immunization postoperatively with NDV-modified ESb tumor cells was also seen in animals which had been inoculated with a plastic adhesive tumor variant, ESb-MP, which has a reduced metastatic capacity and malignancy *in vivo* [8]. The fact that a cross protection was observed (figure 1 (*b*)) indicates that the two tumor lines carry similar tumor-associated transplantation antigens. This is corroborated by our *in vitro* analysis using tumor-specific cytotoxic T lymphocytes [8].

One problem with specific immunotherapy protocols in the ESb model has been the generation of specific immune escape variants [3]. During metastases of ESb cells from a subcutaneous inoculation site immune escape variants developed after about 12 days in their spleens. The variants did not change the expression of the H-2K^d restricting molecules [1] but changed the expression of the TATA so that they could not be recognized by corresponding CTLs. Recently we were able to induce re-expression of the tumor antigen in such immune escape variants by treatment with 5-azacytidine or the mutagen MNNG [2]. We conclude from these experiments that the immune escape variants represent gene regulatory variants rather than true mutants. From the experiments reported here it seems that such immune escape variants once they are established are not susceptible to the immunity induced by immunization with NDV-modified ESb cells. Recognition of a tumor antigen thus seems to be a prerequisite for the systemic anti-tumor effect and the effect against the micrometastases.

That the therapeutic effect observed is related to the establishment of systemic immunity with specificity for the corresponding tumor antigen is further corroborated by the experiments in which we used two unrelated DBA/2 tumors, SL2 and MDAY-D2, to test the specificity at the level of induction of anti-tumor immunity (figure 5) and after establishment of anti-tumor immunity (figure 3). The anti-tumor immunity could be demonstrated at the level of local tumor growth and at the level of overall survival of the tumor cell inoculated animals.

We have begun to analyse the underlying mechanism of the observed anti-metastatic effects. Both in bulk cultures and in limiting dilution cultures, an amplification effect after stimulation with NDV-modified ESb cells was demonstrated [17]. The frequency of tumor-specific CTL-P was significantly increased when animals were immunized with ESb–NDV compared to ESb alone and bulk cultures containing CTL-P were stimulated to higher cytotoxic activity when using ESb–NDV than ESb alone as stimulator cells. From these data we postulate that the postoperative immunization with a vaccine containing the corresponding tumor

antigen and an agent that might function as a second signal for CTL-P activation [16] might mediate a cytotoxicity amplifying effect. CTL-P might first be recruited to the immunogen and might then receive an activation signal, perhaps via involvement of interferon. Following this activation in the periphery, activated tumor-specific CTL or other immune effector T cells similarly activated might recirculate and be able to detect and destroy micrometastases in internal organs.

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References

[1] ALTEVOGT, P., LEIDIG, S., and HECKL-ÖSTREICHER, B., 1984, Resistance of metastatic tumor variants to tumor-specific cytotoxic T lymphocytes not due to defects in expression of restricting major histocompatibility complex molecules in murine cells. *Cancer Research*, **44**, 5305–5313.

[2] ALTEVOGT, P., VON HOEGEN, P., and SCHIRRMACHER, V., 1986, Immunoresistant metastatic tumor variants can reexpress their tumor antigen by treatment with DNA methylation inhibiting agents. *International Journal of Cancer*, **38**, 707–711.

[3] BOSSLET, K., and SCHIRRMACHER, V., 1982, High-frequency generation of new immunoresistant tumor variants during metastasis of a cloned murine tumor line (ESb). *International Journal of Cancer*, **29**, 195–202.

[4] BOSSLET, K., SCHIRRMACHER, V., and SHANTZ, G., 1979, Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. VI. Similar specificity patterns of protective anti-tumor immunity *in vivo* and of cytolytic T cells *in vitro*. *International Journal of Cancer*, **24**, 303–313.

[5] CASSEL, W. A., and GARRETT, R. E., 1965, Newcastle disease virus as an antineoplastic agent. *Cancer*, **18**, 863–868.

[6] CASSEL, W. A., MURRAY, D. A., TORBIN, A. H., OLKOWSKI, Z. L., and MOORE, M. E., 1977, Viral oncolysate in the management of malignant melanoma. I. Preparation of the oncolysate and measurement of immunologic responses. *Cancer*, **40**, 672–679.

[7] CASSEL, W. A., MURRAY, D. R., and PHILLIPS, H. S., 1983, A phase II study on the postsurgical management of stage II malignant melanoma with a Newcastle disease virus oncolysate. *Cancer*, **52**, 856–860.

[8] FOGEL, M., ALTEVOGT, P., and SCHIRRMACHER, V., 1983, Metastatic potential severely altered by changes in tumor cell adhesiveness and cell surface sialylation. *Journal of Experimental Medicine*, **157**, 371–376.

[9] HEICAPPELL, R., SCHIRRMACHER, V., VON HOEGEN, P., AHLERT, T., and APPELHANS, B., 1986, Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. I. Parameters for optimal therapeutic effects. *International Journal of Cancer*, **37**, 569–577.

[10] ITO, Y., NAGAI, Y., and MAENO, K., 1982, Interferon production in mouse spleen cells and mouse fibroblasts (L cells) stimulated by various strains of Newcastle disease virus. *Journal of Genetic Virology*, **62**, 349–352.

[11] KERBEL, R. S., TWIDDY, R. R., and ROBERTSON, D. M., 1978, Induction of a tumor with greatly increased metastatic growth potential by injection of cells from a low-metastatic H-2 heterozygous tumor cell line into an H-2 incompatible parental strain. *International Journal of Cancer*, **22**, 583–594.

[12] KOBAYASHI, H., 1979, Viral xenogenization of intact tumor cells. *Advances in Cancer Research*, **30**, 279–299.

[13] RUSSELL, P. H., and ALEXANDER, D. J., 1983, Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. *Archives of Virology*, **75**, 243–253.

[14] SCHIRRMACHER, V., SHANTZ, G., CLAUER, K., KOMITOWSKI, D., ZIMMERMANN, H.-P., and LOHMANN-MATTHES, M. L., 1979, Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. I. Tumor invasiveness *in vitro* and metastases formation *in vivo*. *International Journal of Cancer*, **23**, 233–244.

[15] SCHIRRMACHER, V., BOSSLET, K., SHANTZ, G., CLAUER, K., and HÜBSCH, D., 1979, Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. IV. Antigenic differences between a metastasizing variant and the parental tumor line revealed by cytotoxic T-lymphocytes. *International Journal of Cancer*, **23,** 245–252.

[16] SCHIRRMACHER, V., AHLERT, T., HEICAPPELL, R., APPELHANS, B., and VON HOEGEN, P., 1986, Successful application of non oncogenic viruses for antimetastatic cancer immunotherapy. *Cancer Reviews*, **5** (in the press).

[17] VON HOEGEN, P., and SCHIRRMACHER, V., 1986, Specific T cell responses to chemically induced tumors analysed by limiting dilution analysis. Viral xenogenization increases the frequency of cytotoxic T lymphocyte precursors specific for the tumor associated transplantation antigen. (Submitted for publication.)

[18] WOLF, A., BARFOOT, R. K., and JOHNSON, R. A., 1972, Xenogeneic recognition of tumor specific plasma membrane antigens derived from murine lymphoma cells. *Immunology*, **22,** 485–491.