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IMMUNOTHERAPY WITH IRRADIATED TUMOUR CELLS AND BCG IN EXPERIMENTAL OSTEOSARCOMA

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The effects of immunotherapy with irradiated tumour cells and BCG were studied in a non-metastasizing variety of the Dunn osteosarcoma transplantable in mice. Experimental animals which had been preimmunized with three injections of 0.7 to 1.4×10^6 irradiated tumour cells each 1 to 3 weeks before administration of 1×10^6 living tumour cells, showed a tumour incidence of 23 per cent. This was significantly ($P < 0.005$) lower than the 92 per cent tumour incidence in the control animals.
Non-specific immunotherapy with BCG given subcutaneously at a dose of 1.0 mg of dry-weight bacterial mass three times at 3-week intervals was found to have no protective effect against the osteosarcoma. The tumour incidence was 90 per cent for BCG-treated and 94 per cent for control animals.
The osteosarcomas were studied light and electron microscopically and also with regard to the histochemical alkaline phosphatase activity. No structural difference was found between the tumours of the various groups.
The demonstrated immunotherapeutic response is in contrast to the low degree of immunogenicity of the osteosarcoma, which we will report elsewhere.

Key words: experimental; immunotherapy; osteosarcoma, transplantable

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Studies of experimental osteosarcoma may give valuable information, also from the clinical point of view, although there are inherent difficulties in animal models. In a previous study (Larsson et al. 1979), we have structurally characterized a transplantable osteosarcoma, a tumour variety obtained from the original Dunn osteosarcoma (Dunn & Anderwont 1963). The more malignant variant with metastatic potential shows striking similarities with human osteosarcoma (cf. Ghanta et al. 1978). There are also varieties of the Dunn osteosarcoma which do not cause pulmonary metastases. Because of their osteoid and alkaline phosphatase production, as well as their histopathological appearance, these tumours nevertheless fulfil the criteria for osteosarcoma (cf. Urist et al. 1977, Larsson et al. 1979).

The Dunn osteosarcoma has a low degree of antigenicity as reported by Ghanta et al. (1978). Thus, even in mice preimmunized with osteosarcoma cells pulmonary metastases occur regularly after tumour implantation. No suppression of tumour growth could be observed upon non-specific immunotherapy with *C. granulosum* and *C. parvum*. As one of the factors which might govern metastatic spread is the degree of antigenicity, it was considered to be of interest to examine the effects of immunotherapy with irradiated tumour cells and BCG in animals with the non-metastasizing type of Dunn osteosarcoma. The light microscopical and ul-

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trastructural characteristics of the tumour are also reported.

MATERIAL AND METHODS

1. *Preimmunization with irradiated tumour cells.* A total of 27 female CBA mice approximately 3 months old were randomly divided into one control group of 13 mice, and one experimental group of 14 animals. Tumour cell suspensions of the osteosarcoma described previously (Larsson et al. 1979) were prepared by collagenase treatment of minced tumour tissue in 1 per cent Dulbecco's medium at 37°C for 5 hours. The cells were kept in Tyrode's solution after washing three times. Dead tumour cells were prepared by irradiating cell suspensions with 15,000 rad ^{60}Co (125 rad/minute). Suspensions of pooled dead cells from the liver, kidney and spleen of normal CBA mice were prepared in the same way as the tumour cells.

The experimental animals were preimmunized by subcutaneous injections of 1.4, 1.4 and 0.7×10^6 irradiated tumour cells per animal, given 3, 2 and 1 week before the subcutaneous administration of 1.0×10^6 living tumour cells per animal. The control animals were treated in parallel, receiving the same quantities of irradiated pooled cells from liver, kidney and spleen and the 1.0×10^6 living tumour cells per animal.

2. *Immunotherapy with BCG (Bacillus Calmette – Guérin).* Freeze-dried Danish BCG vaccine* was injected subcutaneously into 21 CBA mice at a dose of 1.0 mg of dry-weight bacterial mass in 0.2 ml of Sauton solution. This procedure was repeated twice at 3-week intervals. A control group of 18 animals was given parallel injections of the Sauton solution with no BCG added. Fourteen days after the last injection 1.3×10^6 living tumour cells per animal were administered subcutaneously to all the experimental and control mice.

The occurrence of tumour was determined in both experiments by palpation of each animal under light ether anaesthesia at the site of the tumour cell injection (dorsum). The examination was performed once a week. After an observation period of 32 days all the animals of the two experimental groups were sacrificed and all the tumour tissue meticulously dissected out. The wet weight of each tumour was recorded immediately on a Mettler balance, using weighing bottles. Thereafter, an autopsy of each animal was performed and tissue which could be suspected to represent metastasis was removed for histopathological examination.

* Obtained from Statens Seruminstitut, Amager Boulevard 80, 2300 Copenhagen S, Denmark, by courtesy of Dr. K. Bunch-Christensen.

Light microscopy and histochemistry. Specimens taken for light microscopic examination were fixed for 24 hours in 10 per cent neutral formalin. Paraffin embedded sections were stained with haematoxylin and eosin, van Gieson's stain, periodic acid Schiff (P.A.S.), Laidlaw's silver stain and phosphotungstic acid haematoxylin. Histochemical demonstration of alkaline phosphatase was carried out on fresh frozen sections, using the method described by Barka & Anderson (1963).

Electron microscopy. The specimens taken for ultrastructural study were fixed in 2.5 per cent glutaraldehyde in 0.34 M Veronal acetate buffer adjusted to pH 7.4, followed by postfixation in 1 per cent osmium tetroxide in the same buffer. Embedding was carried out in Epon 812, and thick sections stained with toluidine-blue were used for identification of suitable areas for the thin sections which were stained with uranyl acetate and lead citrate prior to examination in a Siemens Elmiskop 1A or 101.

Statistical analysis. A Chi-square test was used.

RESULTS

1. *Preimmunization with irradiated tumour cells.* One experimental animal died from an unrelated disease and was excluded. Among the 13 control animals, tumour occurred in 12, i.e. 92 per cent. Three of the 13 experimental mice developed osteosarcoma, i.e. 23 per cent. The difference between the groups was statistically significant ($P < 0.005$). The wet weight of the tumours is shown in Table 1. Although the tumour incidence in the preimmunized animals was considerably lower than in the controls, there was no significant difference as to tumour weight. None of the animals had metastases.

2. *Immunotherapy with BCG.* Among the 18 control animals, osteosarcoma developed in 17, i.e. 94 per cent. Tumour occurred in 19 out of 21 of the BCG-treated animals, i.e. 90 per cent. The mean value of the wet weight of the tumour in the BCG-treated animals was somewhat lower than that of the controls (Table 1). The difference was not statistically significant, however. There were no metastases in either of the groups.

Table 1. Incidence of osteosarcoma and tumour weights

Treatment	Incidence (per cent)	*P*	Tumour weight (gram)	*P*
Irradiated tumour cells	23	$P<0.005$	2.83±1.50	n.s.
Controls	92		2.88±1.65	
BCG given subcutaneously	90	n.s.	1.49±1.27	n.s.
Controls	94		2.21±1.62	

Light and electron microscopic and histochemical findings

No obvious structural differences were found between the tumours in the control animals on the one hand and those in the animals subjected to immunotherapeutic treatment on the other hand. Nor were any obvious structural differences observed between the tumours in the animals preimmunized with irradiated tumour cells and those subjected to immunotherapy with BCG. Thus, all tumours showed a similar microscopic appearance. Light microscopy disclosed that the tumours were mainly composed of fibroblast-like cells which were irregular or spindle-shaped. Their nuclei were irregular or oval and exhibited one or more, fairly large, rather distinct nucleoli. The nuclear membranes were distinct. An average amount of moderately or lightly staining cytoplasm was found. Generally these cells exhibited a moderate degree of atypism and an average number of typical and atypical mitotic figures (Figure 1).

A sparse number of osteoblast-like cells were found in the tumours. They were usually irregular and possessed rounded, oval or irregular nuclei and one or more moderately distinct nucleoli. The nuclear membranes were distinct. The cytoplasm was fairly well developed and lightly stained. The cell membranes were rather indistinct. Typical and atypical mitotic figures were also found in this kind of cells, often in large quantities.

Areas of necrosis or bleeding were frequently observed in the tumours. Osteoid was seen in sparse amounts, whereas no chondromatous tissue was present. Generally, the tumours possessed a moderately rich vascularization. At the periphery of the tumours there was often a diffuse infiltrative growth into surrounding soft tissues (Figure 2).

Alkaline phosphatase activity was revealed histochemically in all tumours.

Electron microscopy verified the light microscopic observations described above. In addition, the ultrastructural studies disclosed that the *fibroblast-like cells* possessed a rather prominent rough-surfaced endoplasmic reticulum, and a large number of free ribosomes. There was slightly electron dense, flocculent material in some cisterns of the endoplasmic reticulum. In most fibroblast-cells the Golgi complex was small. The mitochondria were moderately large and often rounded or elongated. The cell membranes were usually irregular.

The *osteoblast-like cells* exhibited a well-developed rough-surfaced endoplasmic reticulum, often containing amorphous masses of low density. The Golgi complex was moderately developed. The mitochondria were small or medium-sized and possessed distinct cristae and a moderate matriceal density. Thin fibrils were observed in many osteoblast-like cells, and in a few cells there were bundles of such fibrils. Collagen fibres were encountered extracellularly.

DISCUSSION

Tumour associated antigens not present in normal cells have been demonstrated in animal neoplasms (Old & Boyse 1966, Hellström & Hellström 1969) and in human tumours (Klein 1966, Hellström et al. 1968). The cell membrane is the site of antigens associated with sarcoma (Morton et al. 1969), and antibodies against tumour cells have been demonstrated in osteosarcoma patients. Treated osteosarcoma patients showing no symptoms of disease for several years carry high levels of cytotoxic antibodies against the neoplasm (Morton & Malmgren 1968). The cytotoxic antibody levels were found to be inversely correlated with the presence of tumour.

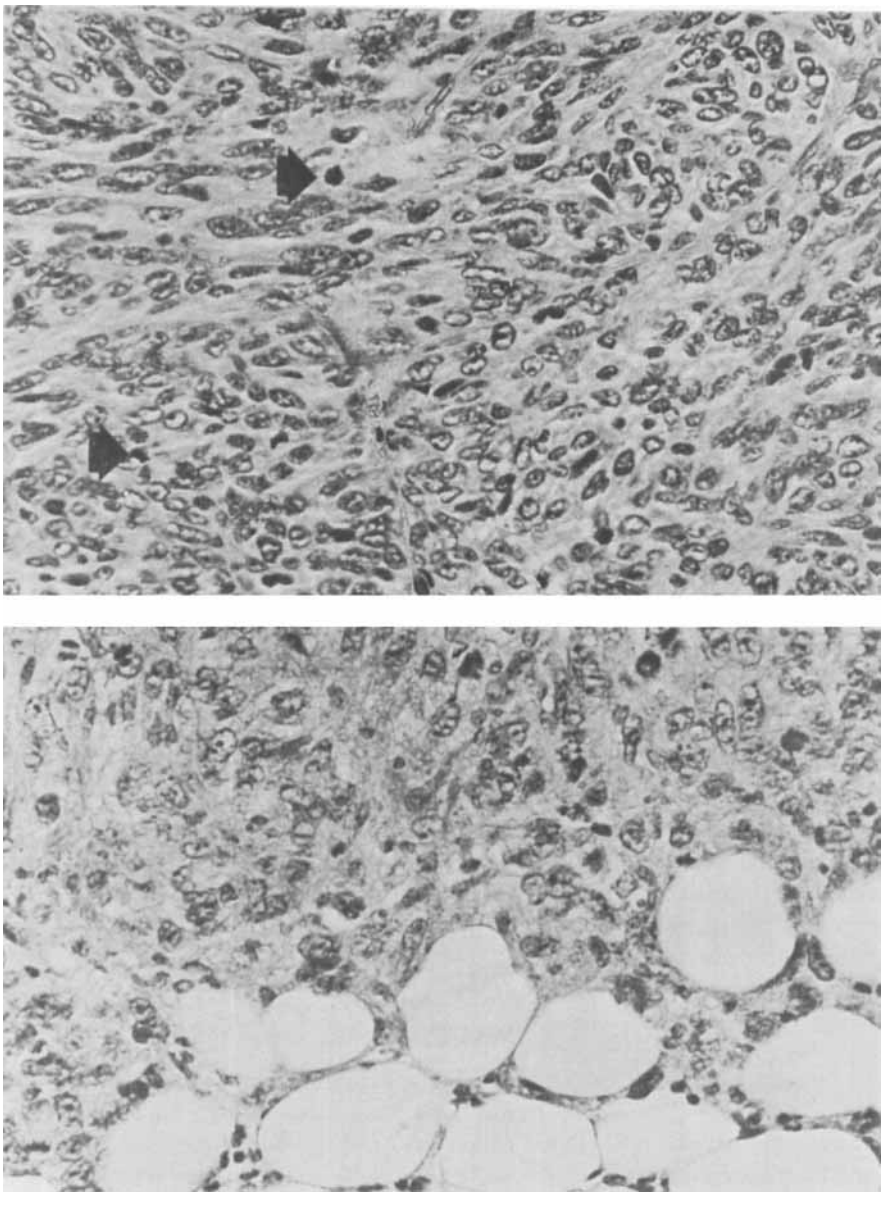

Figure 1. Photomicrograph of an osteosarcoma from a mouse subjected to immunotherapy with BCG, demonstrating closely packed irregular, oval, or spindle-shaped tumour cells with moderate polymorphism. Mitotic figures are seen (arrows). Haematoxylin & eosin ×290.

Figure 2. Photomicrograph of an osteosarcoma from a mouse pre-immunized with irradiated tumour cells, demonstrating infiltrative growth in fatty tissue. Haematoxylin & eosin ×320.

BCG organisms (Mycobacterium tuberculosis bovis of the strain of Calmette and Guérin) are effective in causing an immune response by a non-specific stimulation of the reticuloendothelial system (Old et al. 1961). Therefore, BCG has been administered to both tumour-bearing animals (cf. Bansal & Sjögren 1973) and humans (cf. Mathé et al. 1973). However, the

anti-tumour effect of BCG is disputed. Osteosarcoma induced in mice by radiostrontium shows a weak tumour-specific antigenicity (Moore & Williams 1972). Nevertheless, we could show that BCG treatment had no significant effect on the occurrence of and the mortality in ^{90}Sr-induced osteosarcoma (Larsson et al. 1977). The absence of an effect of BCG treatment in the present study can be explained by the very low antigenicity of the tumour which we have demonstrated in a subsequent study (Carlson et al., to be published).

The immunological defence against neoplasms may involve thymus dependent lymphocytes causing direct cellular killing and, furthermore, non-specific, thymus independent lymphocytes with antibody evoked cytotoxicity (cf. Perlmann & Holm 1969). In most current work in experimental tumour immunotherapy highly antigenic tumours have been used and the results cannot therefore be translated to man. Interestingly, a specific immunotherapeutic response could be evoked in the present study despite the very low antigenicity of the tumour. Thus, in a subsequent study we have shown that CBA mice bearing this osteosarcoma do not develop detectable cell mediated nor humoral immune response against the tumour (Carlson et al., to be published). However, adult thymectomy affected both the tumour occurrence which became significantly reduced, and the cell mediated cytotoxic activity against this tumour which became significantly increased (Carlson et al., to be published). Thymosin treatment of thymectomized mice restored both parameters to the level of untreated animals but did not affect nonthymectomized animals at all. The results can be explained by a decrease in T-lymphocyte suppressor activity induced by the adult thymectomy (Monier et al. 1970).

The specific immunotherapeutic response evoked by irradiated tumour cells in the present study can not be readily explained in view of our results described above. The metastasizing variety of the Dunn osteosarcoma has low-grade antigenicity and immunotherapy with irradiated or living tumour cells does not evoke a detectable specific response (Miller et al. 1976, Ghanta et al. 1978). Further studies of the complex cell mediated and humoral factors involved in tumour defence may shed more light on the resistance to neoplasms and the possibilities of immunotherapy.

REFERENCES

Bansal, S. C. & Sjögren, H. O. (1973) Correlation between changes in antitumour immune parameters and tumour growth in vivo in rats. *Fed. Proc.* **32**, 165.

Barka, T. & Anderson, P. J. (1963) *Histochemistry. Theory, practice and bibliography*, p. 240. Harper and Row, Publ. Inc. New York, Evanstone and London.

Dunn, T. B. & Andervont, H. B. (1963) Histology of some neoplasms and non-neoplastic lesions found in wild mice maintained under laboratory conditions. *J. Natl. Cancer Inst.* **31**, 873–882.

Foley, E. J. (1953) Antigenic properties of methylcholanthrene-induced tumours in mice of the strain of origin. *Cancer Res.* **13**, 835–837.

Ghanta, V. K., Hiramoto, R. N., Weiss, A. B., Caudill, L. & Davis, D. W. (1978) Tumour immunology of experimental osteosarcoma. *Clin. Orthop.* **133**, 246–260.

Glasgow, L. A., Crane Jr., J. L. & Kern, E. R. (1978) Antitumour activity of interferon against murine osteogenic sarcoma cells in vitro. *J. Natl. Cancer Inst.* **60**, 659–663.

Hellström, I., Hellström, K. E., Pierce, G. E. & Young, J. P. S. (1968) Cellular and humoral immunity to different types of human neoplasms. *Nature* **220**, 1352–1354.

Hellström, I. & Hellström, K. E. (1969) Studies on cellular immunity and its serum-mediated inhibition in Moloney-virus induced mouse sarcomas. *Int. J. Cancer* **4**, 587–593.

Klein, G. (1966) Tumour antigens. *Annu. Rev. Microbiol.* **29**, 223–236.

Larsson, S.-E., Lorentzon, R. & Boquist, L. (1977) The effect of immunotherapy with BCG on the development of radiostrontium (^{90}Sr)-induced osteosarcoma. *Acta Pathol. Microbiol. Scand.* [A] **85**, 433–446.

Larsson, S.-E., Lorentzon, R., Lundgren, E. & Boquist, L. (1979) Transplantable osteosarcoma in mice. Structural characterization of a transplantable osteosarcoma obtained in an allogeneic system. *Acta Orthop. Scand.* **50**, 481–489.

Mathé, G., Kanel, M., Dezfulian, M., Halle-Pannenko, O. & Bournt, C. (1973) An experimental screening for "systemic adjuvants of immunity" applicable in cancer immunotherapy. *Cancer Res.* **33**, 1987–1997.

Miller, C. W., DeBlasi, R. F. & Fisher, S. J. (1976) Immunological studies in murine osteosarcoma. *J. Bone Joint Surg.* **58**-A, 312–317.

Moore, M. & Williams, D. E. (1972) Studies on the antigenicity of radiation-induced murine osteosarcomata. *Br. J. Cancer* **26**, 90–98.

Morton, D. L. & Malmgren, R. A. (1968) Human osteosarcomas: immunologic evidence suggesting an associated infectious agent. *Science* **162**, 1278–1280.

Morton, D. L., Malmgren, R. A., Hall, W. R. & Schidlovsky, G. (1969) Immunologic and virus studies with human sarcomas. *Surgery* **66**, 152–161.

Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E. A. & Stochert, E. (1961) The role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* **21**, 1281–1300.

Old, L. J. & Boyse, E. A. (1966) Specific antigens of tumours and leukaemias of experimental animals. *Med. Clin. North Am.* **50**, 901–909.

Perlmann, P. & Holm, G. (1969) Cytotoxic effects of lymphoid cells in vitro. *Adv. Immunol.* **2**, 117–121.

Urist, M. R., Nakata, N., Felser, J. M., Nogami, H., Hanamura, H., Miki, T. & Finerman, G. A. M. (1977) An osteosarcoma cell and matrix retained morphogen for normal bone formation. *Clin. Orthop.* **124**, 251–266.

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