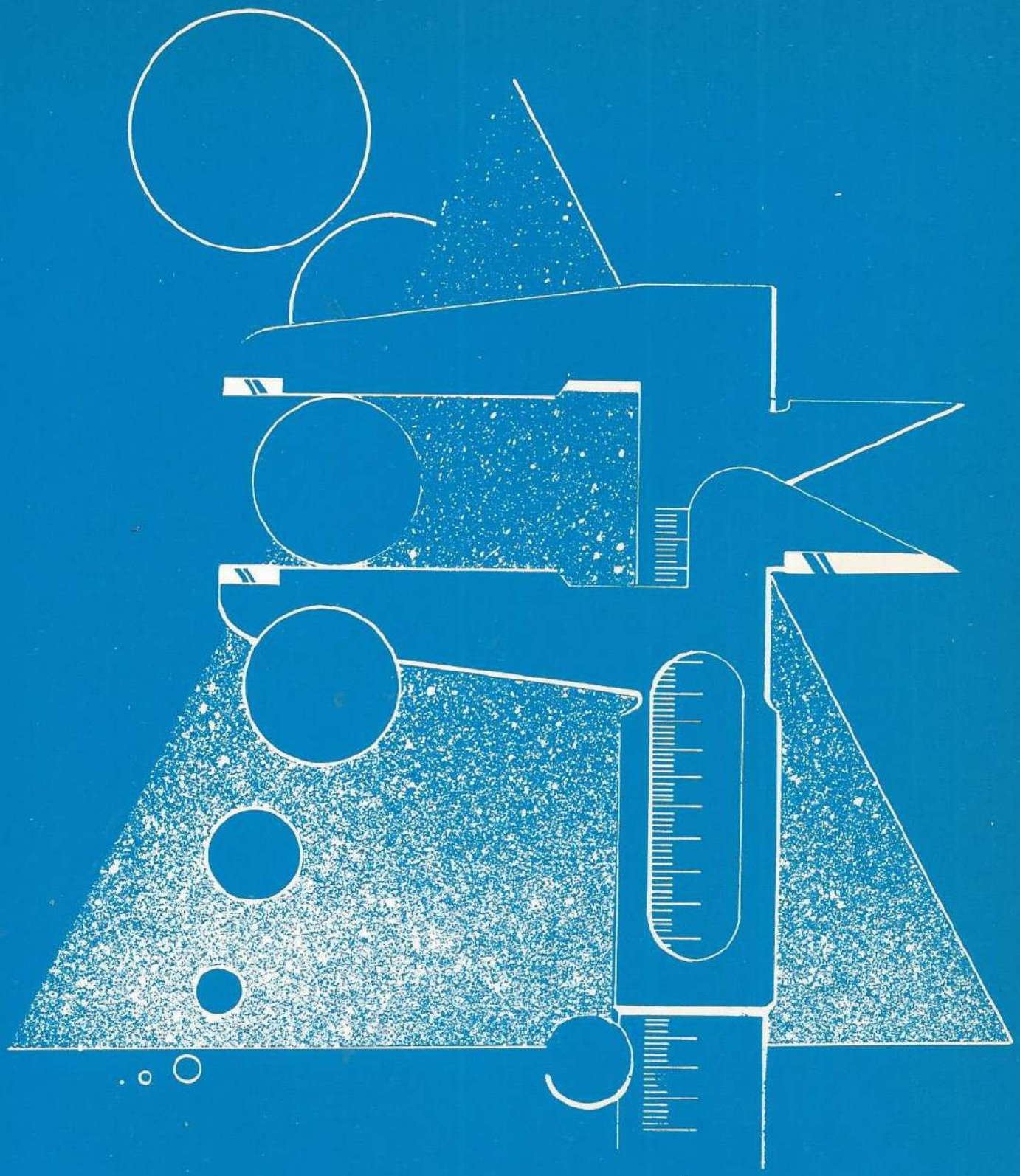

Transplantation of Human Head and Neck Cancer Xenografts in Athymic Nude Mice

Boudewijn J.M. Braakhuis

TRANSPLANTATION OF HUMAN HEAD AND NECK CANCER XENOGRAFTS IN ATHYMIC NUDE MICE

A potential screening model
for anticancer drugs

Het onderzoek beschreven in dit proefschrift werd gesteund door het Koningin Wilhelmina Fonds (KWF)

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door

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geboren te Hoorn

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Ter nagedachtenis van mijn vader

Voor mijn moeder

Voor Christa en onze kinderen
Pieter en Leonie

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CONTENTS

LIST OF ABBREVIATIONS

BLEO	bleomycin
CDDP	cis-diamminedichloroplatinum (II)
CY	cyclophosphamide
DHFR	dihydrofolate reductase
E:T	effector to target ratio
i.p. (ip)	intraperitoneal
i.v. (iv)	intravenous
LDH	lactate dehydrogenase
MTX	methotrexate
NCI	National Cancer Institute
NK	natural killer
s.c. (sc)	subcutaneous
SPF	specific pathogen free
5-aza-dCyd	5-aza-2'-deoxycytidine
5'dFUR	5'-deoxy-5-fluorouridine
5-FU	5-fluorouracil

CHAPTER 1

GENERAL INTRODUCTION

Chemotherapy in head and neck cancer

In the western hemisphere head and neck cancers account for 5-14% of all malignancies. The frequency depends on the country, being highest in Southern Europe (1). Surgery and radiotherapy are the most effective tools but seem to have reached a plateau in their ability to cure head and neck cancer. Especially patients with stage III and IV disease continue to be a therapeutic problem. The rationale to integrate chemotherapy into combined modality treatment in these advanced stages is clear. However, the value of adjuvant chemotherapy in the treatment of patients with advanced head and neck cancer has yet to be proven through randomized clinical trials.

A few cytotoxic drugs have been demonstrated to be active in head and neck cancer; methotrexate, bleomycin, cisplatin and cyclophosphamide give objective responses in 30-40% of patients (2, 3). The combination of 5-fluorouracil and cisplatin was found to be extremely effective when used as an initial therapy before surgery and/or radiotherapy, producing response rates of 90% (4). However, this type of combination chemotherapy is associated with much toxicity. Therefore the need for new drugs with less toxicity remains. The question as to which drugs are to be selected for phase II trials in head and neck cancer patients is of paramount importance in this regard.

Selection of new drugs

Until now the selection of drugs for phase II trials in patients with head and neck cancer was based on the activity of a given drug in other tumour types or solely on the activity in murine tumours (mostly leukaemias) used for the last decade by the National Cancer Institute (NCI, USA) (5, 6). During the last two years the role of animal tumour models has

been extensively discussed (7, 8), because it is a well-known fact that the selection of drugs based on the animal tumour models very seldom leads to clinically active drugs (9). A lower priority has been given to the animal tumour models since the NCI has taken the decision to test drugs in vitro on human tumour cell lines in a so-called disease-oriented approach (10). In vitro screening also has disadvantages, because important factors including tumour-host interaction, vascularization, drug metabolism and distribution are lacking.

Tumour xenografts

An attractive possibility is to study human tumours in vivo as xenografts in experimental animals. Some success in xenografting has been achieved with newborn rats and mice, with immunosuppressed animals or on privileged sites as the brain, the anterior chamber of the eye or the cheek pouch of the hamster. Since the nude mice became available in the early seventies (11) the majority of investigators have used this animal as host. Some investigators (12) have used normal mice after depressing their immune system by total body irradiation and thymectomy. The disadvantage of the use of these animals is that after a period of 6 to 10 weeks the tumours regress due to a recovery of the immune system. In these artificially depressed animals long term experiments are impossible.

Nude mice

Athymic nude mice have a poorly developed thymus (13) leading to a very low number of lymphocytes with T-cell-markers (14). The antibody production is lower (13) and the number of natural killer cells (15) and macrophages (16) is increased in these animals when compared to normal mice. The impaired cell mediated immunity makes the acceptance of foreign tissues possible, but the animal is also very sensitive to viral, bacterial and parasitic infections. To ensure a normal lifespan these animals should be kept in a "specific pathogen free " environment (17), which means that the animals are to be housed in cages which give protection against microorganisms, located in a temperature- and humidity controlled environment. Moreover, it is necessary that all materials that come into contact with

the mice are sterilized before use. Personnel should be well trained, and wear uniforms, masks and gloves.

Tumour xenografts in nude mice

The first successful xenografting of tumours was reported in 1969 by Rygaard and Povlsen (18). Since then, a variety of human tumour types has been transplanted in this animal (19). Concerning the characteristics of the human tumours maintained in these animals, it has been established that the xenografts retain their human nature during serial passage. Human lactate dehydrogenase (20, 21), $ß_2$-microglobulin (22) and carcinoembryonic antigen (23) can be determined in the serum of nude mice. Cytogenetic studies have also confirmed the human nature of xenografts (24).

In most cases the histology does not change during serial passage (12). This indicates that xenografted cells still have the possibility to differentiate. Studies with colon carcinomas in immunosuppressed animals have shown that during the adaptation of a xenograft in the nude mouse the majority of the tumour cells die. The only cells that survive are undifferentiated cells that give rise to a differentiated carcinoma (25). Thus, the nude mouse provides an environment that enables the tumour cell to differentiate to a certain degree. Some authors report a change in the degree of differentiation during serial passage. Giovanella et al. (26) found an increase in the degree of differentiation in 25% of tumours taken from various parts of the body. Also dedifferentiation has been noted for lung (27) and breast carcinomas (28). It must be assumed that the human tumour in the murine host is still heterogeneous like it is in the patient and that different subpopulations occur in a single tumour. Flow cytometric studies showed the evolution of a subpopulation of cells taking place during passaging (29-32). It is important to investigate the microscopical appearence of the xenografted tumours during passaging, because changes in histology may be parallelled by changes in the sensitivity to treatments. Another reason for continuing histopathological investigations is to detect possible murine tumours induced by the human xenografts. Lymphomas (33) and sarcomas (34) can be observed adjacent to the human tumours which can be easily overgrown by these mouse tumours.

Considering growth kinetics it is assumed that the xenografts are

different from the tumours in the patient. Most xenografted tumours grow faster than the tumours in the human host (12) and in a number of papers a decrease of the tumour volume doubling time is reported when the number of passages increased. (27, 31, 33, 36). Cell kinetic studies have indicated that the number of cells in the S-phase may increase (27) or cell loss decrease with increasing passage (31, 36).

Tumour take in nude mice

The take rate of human tumours varies with tumour type (19). For instance for breast and prostatic carcinomas transplantable tumour lines can be established only occasionally (37). Concerning head and neck cancer a take in the first passage is generally observed in 29 to 36% of the cases (38-41). Recently, Elprana et al. reported a take with 10 out of 13 tumours attempted (42). Wennerberg et al. found that serial passaging was possible in half of the cases that initially took (38). It is an intriguing question why only certain tumours grow in nude mice. Non-immunological factors might play a role in this respect such as the availability of growth factors, the viability of the specimen at the time of transplantation or the hormonal status of the animal. Also immunological mechanisms may account for the lack of growth of tumours as indicated by the increase in success rate for transplantation after total body irradiation (43) or antilymphocyte antiserum treatment (44). Studies to enhance the take rate of poorly growing tumours are to be encouraged.

Sensitivity testing

In spite of the fact that for a great variety of human tumour lines in nude mice both the biological characteristics and the response to anticancer drugs have been established, the clinical relevance of the model is not yet clear. There is evidence that suggests that xenografts of a given tumour type are sensitive to drugs that are clinically active (12, 45-47). Also, it is reported that xenografts retain the same sensitivity as the individual patient from whom the original tumour was derived. (35, 48, 49). As in patients, the xenograft lines of a particular tumour type have significant differences in sensitivity to a specific drug (48, 50). Since it

takes more than a couple of months to evaluate a drug, the model is of limited value for the individual patient. The validity of the model will lie almost exclusively in the field of screening of new agents. Because of the aforementioned variability in tumour response a panel of tumours of a given tumour type is needed for an accurate evaluation of activity (51).

Objectives of the study

There is a need for new cytotoxic drugs in head and neck squamous cell carcinoma . The question which drugs are to be tested in phase II clinical trials is very important. We investigated whether a preclinical model could be of value in the selection of agents for clinical studies. Because of the promising results obtained with tumour xenografts in nude mice we decided to use this model as a screening model for new agents. Therefore we have designed a study with the following objectives:

1. Establishment of human head and neck cancer xenografts lines in nude mice.
2. Comparison of the characteristics of the tumour lines and the original patient tumours.
3. Comparison of the effect of conventionally used anticancer drugs in the model with the known clinical response. If possible the response of the xenograft to a given drug was compared with the response to that drug in the patients from whom the tumour was derived.
4. Evaluation of the activity of new agents in the tumour lines.
5. Definition of the role of the nude mouse model for the selection of new agents in head and neck cancer.

In this thesis various aspects of the growth of human head and neck tumours in athymic nude mice will be discussed. In chapter 2 is reported on the possibility to grow human head and neck tumours in nude mice and on the characteristics that make a tumour more likely to take. In chapter 3 the further suppression of the immune system is discussed in an attempt to increase the take of tumours. In chapters 4 to 7 we describe the results with various anticancer drugs tested in this model. Results are presented for new and conventional drugs.

REFERENCES

1. Manual of clinical oncology. Edited under the auspices of the International Union Against Cancer. 3rd edition. Berlin: Springer-Verlag, 1982: 150.
2. Taylor SG. Head and neck cancer. In: Pinedo HM, Chabner BA, eds. Cancer Chemotherapy, Annual 6. Amsterdam: Elsevier, 1984: 285-298.
3. Carter SK. The chemotherapy of head and neck cancer. Semin Oncol 1977; 4: 413-424.
4. Weaver A, Fleming S, Ensley J and 5 others. Superior clinical response and survival rates with initial bolus of cisplatin and 120 hour infusion of 5-fluorouracil before definitive therapy for locally advanced head and neck cancer. Am J Surg 1984; 148: 525-529.
5. Goldin A, Venditti JM, MacDonald JS, Muggia FM, Henney JE, DeVita VT. Current results of the screening program at the division of cancer treatment, National Cancer Institute. Eur J Cancer 1981; 17: 129-142.
6. Staquet MJ, Byar DP, Green SB, Rozencweig M. Clinical predictivity of tranplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three stage-strategy. Cancer Treat Rep 1983; 67: 753-765.
7. The use of rodent tumors in experimental cancer chemotherapy. Conclusions and recommendations from an international workshop. Cancer Res 1985; 45: 6541-6545.
8. Martin DS, Balis ME, Fisher B and 13 others. Role of murine tumor models in cancer treatment research. Cancer Res 1986; 46: 2189-2192.
9. Hoth DF, Marsoni S, Rubenstein LV and 4 others. The NCI drug screen: correlation between preclinical and clinical activity 1970-1985. Abstract 5th NCI-EORTC symposium on new drugs in cancer therapy, Amsterdam, 1986.
10. Boyd M, Shoemaker R, Alley M and 7 others. New NCI disease-oriented drug screening program. Abstract 5th NCI-EORTC symposium on new drugs in cancer therapy, Amsterdam, 1986.
11. Povlsen CO, Rygaard J. Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant nude. A study of nine consecutive transplantations. Acta Path Microbiol Scand (A) 1971; 79: 159-169.
12. Steel GG, Peckham MJ. Human tumour xenografts: a critical appraisal. Br J Cancer 1980; 41, Suppl. IV: 133-141.
13. Rygaard J. Immunodeficiency in nude mice. In: Bastert GBA et al., eds. Thymusaplastic nude mice and rats in clinical oncology. Stuttgart: Fischer Verlag, 1981: 3-13
14. MacDonald HR. Phenotypic and functional characteristics of "T-like" cells in nude mice. In: Sordat B, ed. Immune-deficient animals. Basel: Karger, 1984: 2-6.
15. Schultz RM. Factors limiting tumoricidal function of interferon-induced effector systems. Activated macrophages and natural killer cells. Cancer Immunol Immunother 1981; 10: 61-66.
16. Sharp AK, Colston MJ. The regulation of macrophage activity in congenitally athymic mice. Eur J Immunol 1984; 14: 102-105.
17. Ediger R, Giovanella BC. Current knowledge of breeding and mass production of the nude mouse. In: Fogh J, Giovanella BC, eds. The nude mouse in experimental and clinical research. New York: Academic Press. 1978: 16-28.

18. Rygaard J, Povlsen CO. Heterotransplantation of a human malignant tumour to "nude" mice. Acta Path Microbiol Scand 1969; 77: 758-760.
19. List of human tumors transplanted to nude mice. In: Nomura T et al., eds. Proceedings 2nd workshop on nude mice. Tokyo: University of Tokyo Press. 1977: 587-595
20. Heckl W, Fogh J. Human lactic dehydrogenase as a marker for monitoring the growth of prostatic carcinoma in nude mice. Expl Cell Biol 1986; 54: 34-39.
21. Pesce AJ, Bubel HC, DiPersio L, Michael JG. Human lactic dehydrogenase as a marker for human tumor cells grown in athymic mice. Cancer Res 1977; 37: 1998-2003.
22. Wennerberg J, Alm P, Lodgberg L, Tropé C. $ß_2$-microglobulin in squamous cell carcinomas of the head and neck and in tumours heterotransplanted into nude athymic mice. Acta Otolaryngol 1984; 98: 335-342.
23. Staab AJ, Anderer FA. Growth of human colonic adenocarcinoma and development of serum CEA in athymic mice. Br J Cancer 1982; 46: 841-847.
24. Visveldt J, Povlsen CO, Rygaard J. Chromosome analyses of human tumours following heterotransplantation of the nude mouse mutant nude. Acta Pathol Microbiol Scand (A) 1972; 80: 169-176.
25. Barkla DH. Tutton PJM. Temporal morphologic changes in human colorectal carcinomas following xenografting. Am J Pathol 1983; 110: 315-321.
26. Giovanella BC, Stehlin JS, Fogh J, Sharkey FE. Serial transplantation of human malignant tumors in nude mice and their use in experimental chemotherapy. In: Houchens DP, Ovejera AA, eds. Proceedings of the symposium of the use of athymic (nude) mice in cancer research. Stuttgart/New York: Fischer, 1978: 163-179.
27. Mattern J, Haag D, Wayss K. Volm M. Growth kinetics of human lung tumours in nude mice. Expl Cell Biol 1981; 49: 34-40.
28. Bailey MJ, Ormerod MG, Imrie SF and 4 others. Comparative functional histopathology of human breast carcinoma xenografts. Br J Cancer 1981: 43; 125-134.
29. Baisch H, Otto U, Kloeppel G. Long-term serial transplantation of 30 different human renal cell carcinomas into NMRI (nu/nu) mice: flow cytometric, histologic, and growth studies. J Natl Cancer Inst 1986; 76; 269-276.
30. Vindelov LL, Spang-Thomsen M, Visveldt J, Povlsen CO, Jensen G, Nissen NI. Clonal evolution demonstrated by flow cytometric DNA Analysis of a human colonic carcinoma grown in nude mice. Expl Cell Biol 1982; 50: 216-221.
31. Wennerberg J. Changes in growth pattern of human squamous-cell carcinomas of the head and neck during serial passages in nude mice. Int J Cancer 1984; 33: 245-250.
32. Russel PJ, Raghavan D, Gregory P and 6 others. Bladder cancer xenografts a model for tumor cell heterogeneity. Cancer Res 1986; 46: 2035-2040.
33. Baird SM, Beattie GM, Lannom RA and 3 others. Induction of lymphoma in antigenically stimulated athymic mice. Cancer Res 1982; 42: 198-206.
34. Beattie GM, Knowles AF, Jensen FC, Baird SM, Kaplan NO. Induction of sarcomas in athymic mice. Proc Natl Acad Sci USA 1982; 79: 3033-3036.
35. Fiebig HH, Widmer KH, Fiedler L, Wittekind C, Löhr GW. Development and characterization of 51 human tumor models for large bowel, stomach and esophageal cancers. Dig Surg 1984; 1: 225-235.
36. Houghton JA, Taylor DM. Growth characteristics of human colorectal

tumours during serial passage in immune-deprived mice. Br J Cancer 1978; 37: 213-223.
37. Reid LCM, Shin S. Transplantation of heterologous endocrine tumor cells in nude mice. In: Fogh J, Giovanella BC, eds. The nude mouse in experimental and clinical research. New York: Academic Press, 1978; 313-351.
38. Wennerberg J, Tropé C, Björklund A. Heterotransplantation of human head and neck tumours into nude mice. Acta Otolaryngol (Stockh) 1983; 95: 183-190.
39. Lindenberger J, Ganzer U, Fortmeyer HP. Die Heterotransplantation bösartiger Kopf- und Halsgeschwülste auf thymusaplastische nackte Mäuse. Arch Otorhinolaryngol 1978; 220: 117-128.
40. Hill JH, Plant RL, Harris DM and 4 others. The nude mouse xenograft system: a model for photodetection and photodynamic therapy in head and neck squamous cell carcinoma. Am J Otolaryngol 1986; 7: 17-27.
41. Fantozzi R, Bone R, Fernsten P, Walker L. Upper aerodigestive neoplasm perpetuated in the nude mouse. Laryngoscope 1986; 96: 621-624.
42. Elprana D, Kuijpers W, van den Broek P, Wagener DJT. Growth characteristics of head and neck squamous cell carcinoma in nude mice. Eur J Cancer Clin Oncol 1986; 22: 1211-1222.
43. Prehn LM, Outzen, HC. Primary tumor immunity in nude mice. Int J Cancer 1977; 19: 688-691.
44. Gershwin ME, Ikeda RM, Erickson K, Owens R. Enhancement of heterotransplanted human tumor graft survival in nude mice with antilymphocyte serum and in congenitally athymic-asplenic (lasat) mice. J Natl Cancer Inst 1978; 61:245-248.
45. Giuliani FC, Zirvi KA, Kaplan NO, Goldin A. Chemotherapy of human colorectal tumor xenografts in athymic mice with clinically active drugs: 5-fluorouracil and 1-3-bis-(-2-chloroethyl)-1-nitrosourea (BCNU). Comparison with doxorubicin derivatives: 4'deoxydoxorubicin and 4'-0-methyldoxorubicin. Int J Cancer 1981; 27: 5-13.
46. Azar HA, Fernandez SB, Bros LM, Sullivan JL. Human tumor xenografts in athymic (nude) mice: chemotherapy trials in serially transplanted tumors. Ann Clin Lab Sci 1982; 12: 51-59.
47. Povlsen CO, Jacobsen GK. Chemotherapy of a human malignant melanoma transplanted in the nude mouse. Cancer Res 1975; 35: 2790-2796.
48. Osieka R. Studies on drug resistance in a human melanoma xenograft system. Cancer Treat Rev 1984; 11(A); 85-97.
49. Shorthouse AJ, Smyth JF, Steel GG, Ellison M, Mills J, Peckham MJ. The human tumour xenograft-a valid model in experimental chemotherapy? Br J Surg 1980; 67: 715-722.
50. Nowak K, Peckham MJ, Steel GG. Variation in response of xenografts of colo-rectal carcinoma to chemotherapy. Br J Cancer 1978; 37: 576-584.
51. Bellet RE, Danna V, Mastrangelo MJ, Berd D. Evaluation of a "nude" mouse-human tumor panel as a predictive secondary screen for cancer chemotherapeutic agents. J Natl Cancer Inst 1979; 63: 1185-1188.

CHAPTER 2

THE POTENTIAL OF THE NUDE MOUSE XENOGRAFT MODEL FOR THE STUDY OF HEAD AND NECK CANCER

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ABSTRACT

A total of 129 human head and neck cancers were transplanted subcutaneously in athymic nude mice in order to obtain a series of xenografts. All tumours were derived from previously untreated patients. Initial growth, which was histopathologically confirmed, was observed in 34 cases (26%). Serial passages were successful in 12 of 23 cases (52%). Of 116 squamous cell carcinomas, 30 (26%) showed initial take in the mice. Poorly differentiated squamous cell carcinomas tended to grow more readily than moderately differentiated and well differentiated ones. Material from metastatic lymph nodes tended to have a higher take than material from primary tumours. In general the tumour volume doubling time decreased to 4-6 days when the number of passages increased. Histology of the xenografted tumours showed that transplantation had caused no major changes. No macro-, or microscopic signs of metastasis were observed in any of the mice. The implications of this model for fundamental and applied research are discussed.

INTRODUCTION

Athymic nude mice lack a thymus-dependent immune system and various tumours can be successfully transplanted and serially grown in these animals (1-3). This xenograft model seems promising for the study of tumour cell biology and the evaluation of anticancer therapies. The human origin of the xenografted tumour cells is preserved according to the results of chromosome analysis (4), lactate dehydrogenase isoenzyme analysis (5) and the expression of organ-specific neoantigens (6).

However, the extent to which characteristics of the original tumour are maintained in the xenograft still has to be determined. The transplanted tumour grows faster when the number of passages increased (7, 8). Metastasis and invasion, characteristics of malignant tumour growth are rarely found (9).

The success of initial and serial takes varies among tumour categories (1, 10, 11). Little is known about the take of human head and neck tumours in this model. This study describes a group of 129 head and neck tumours which have been transplanted in nude mice in our laboratory. Take rate and

growth pattern are analysed in primary and serial passages. The question as to whether it is possible to use this model to test anticancer therapies is discussed.

MATERIALS AND METHODS

Animals

Female nude mice (B10.LP/Cpb, 8-10 weeks old) were obtained from the Centraal Proefdieren Bedrijf TNO (Zeist, The Netherlands). The mice were kept in a separate room, provided with a barrier system. The cages were covered with paper filters and all manipulations were conducted under a laminar-flow cabinet (Clean Air, Woerden, The Netherlands). Cages, bedding, food (1210, Hope Farms, Woerden, The Netherlands) and water were autoclaved before use. The water was brought to a pH of 2.8 with 0.1 N HCl and contained no antibiotics. The temperature in the animal room ranged from 24 to 28^{o}C and the humidity from 55 to 65%.

Tumours and methods of implantation.

Only head and neck tumours from previously untreated patients were selected for implantation. Tumour specimens were removed aseptically and collected in ice-cold Hanks' buffered salt solution with 200 U/ml penicillin and 200 ug/ml streptomycin. Slices measuring 3 x 3 x 1 mm were dissected and implanted subcutaneously in the lateral thoracic region on both sides of the animal. Human tumours growing in nude mice and measuring between 800 and 1000 mm^3 were serially transplanted in a similar way.

Tumour take and measurement.

Tumour take was defined as the presence of a histopathologically confirmed tumours of increasing size. When a tumour was capable of surviving more than 4 passages we defined that a tumour line was established. Growth was measured biweekly using vernier calipers. Tumour volume was calculated as length x width x height x 0.5 (12). Tumour growth curves were plotted individually on semilog graph paper.

Histopathology

When the animals had been killed, all internal organs were inspected

macroscopically, special attention being given to the regional lymph nodes. Each organ showing an abnormal macroscopic appearance was excised and examined microscopically. In some mice axillary lymph nodes, kidneys, lungs spleens and sections of small intestine and liver were investigated histologically in order to detect any metastatic tumour foci and histopathological changes. Material for microscopic examination was fixed in 4% neutrally buffered formalin and processed for paraffin embedding. Sections of 5 μm were stained with haematoxylin and eosin. At the end of each subpassage, representative tumour slices were taken for microscopy. Histopathologic comparison was made between the mouse grown tumour and its corresponding original human tumour. Attention was also paid to the degree of differentiation, necrosis and invasion of adjacent mouse tissue. Grades of differentiation were classified according to a three-grade scale: well, moderately and poorly differentiated squamous cell carcinomas. When a tumour had varying degrees of differentiation, it was decided that the less differentiated parts were representative for that tumour.

RESULTS

A total of 129 head and neck tumours derived from previously untreated patients were transplanted into athymic nude mice. Tumour growth in the mouse was observed in 34 cases (26%, Table 1). For squamous cell carcinomas, with 116 tumours the largest group, the initial take rate was 26%. Attempts have been performed to establish tumour lines for these xenografts. So far, this has been successful in 12 of 23 (52%) cases. Two tumour lines were lost after the 5th passage. Poorly differentiated squamous cell carcinomas tended to take better than moderately and well differentiated ones (Table 2). Tumours derived from the hypopharynx grew more readily than those from other mucosal sites (Table 3). However, three of the nine hypopharyngeal tumours were poorly differentiated squamous cell carcinomas and all three tumours took initially. Tumour material from lymph node metastasis tended to show initial growth more often than material from primary tumours (Table 4). The success rate in obtaining tumour lines from metastasized tumours was not increased. Takes were obtained from the same patient for metastasis and the primary tumour of a melanoma. The lymph node metastasis of another patient grew successfully, whereas the primary

Table 1. Take of various types of head and neck tumours in nude mice

Tumour type	No. tumour takes/ transplanted (%)	No. serially grown tumours[#]/attempted (%)
Squamous cell carcinoma	30/116 (26)	11/22* (50)
Salivary gland	2/9 (22)	1/1
Chondrosarcoma	0/1	0/0
Non-Hodgkin lymphoma	0/1	0/0
Mucosal melanoma	2/2	0/0
Total	34/129 (26)	12/23 (52)

*Two lines were lost after the fifth passage.

[#]Serially grown indicates that more than four passages were possible.

Table 2. Take rate of squamous cell carcinomas of the head and neck according to their degree of differentiation

Degree of differentiation	No. tumour takes/ transplanted (%)	No. serially grown tumours[#]/attempted (%)
Poorly	7/13 (54)	3/4 (75)
Moderately	12/44 (27)	3/7 (43)
Well	11/59 (19)	5/11*(45)

*One line was lost after the fifth passage

[#]Serially grown indicates that more than four passages were possible.

tumour, which had been transplanted 3 months earlier, failed to grow. In 4 (HNX-P, -LA, -PV and -TI, Table 5) of 6 evaluable tumour lines the take rate in subsequent passages varied between 30 and 100%. In two lines (HNX-G and -W) a relatively constant take rate of 80-100% was reached after two passages. Two examples of these characteristics are given in Fig. 1. The other 6 tumour lines could not be evaluated because the number of passages

Table 3. Take rate of squamous cell carcinomas according to their localization within the head and neck

Localization	No. tumour takes/ transplanted (%)	No. serially grown tumours[#]/attempted (%)
Oral cavity	13/59 (22)	7/11* (64)
Larynx	6/24 (25)	0/4
Oropharynx	4/16 (25)	0/3
Hypopharynx	5/9 (55)	3/3
Nasopharynx	0/4	0/0
Skin	1/2	1/1
Unknown primary site	1/2	0/0
Total	30/116 (26)	11/22 (50)

*One line was lost after the fifth passage

[#]Serially grown indicates that more than four passages were possible.

Table 4. Take rate of head and neck tumours derived from primary tumours and lymph node and distant metastases

Site	No. tumour takes/ transplanted (%)	No. of serially grown tumours[#]/attempted (%)
Primary	27/116 (23)	10/19* (53)
Lymph node metastasis	6/11 (54)	2/4* (50)
Distant metastasis	1/2	0/0
Total	34/129 (26)	12/23 (52)

*One line was lost after the fifth passage

[#]Serially grown indicates that more than four passages were possible.

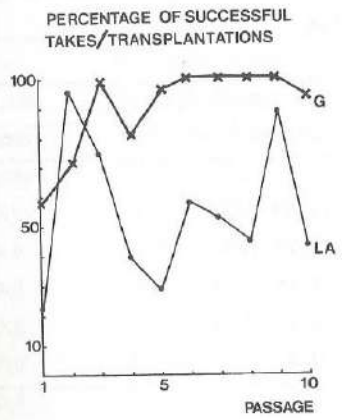

Fig. 1. Percentage of successful takes of attempted transplantation in subsequent passages for the tumour lines HNX-LA and -G. At least 12 tumours were transplanted in each passage. For the sake of clarity HNX- is omitted in the figure.

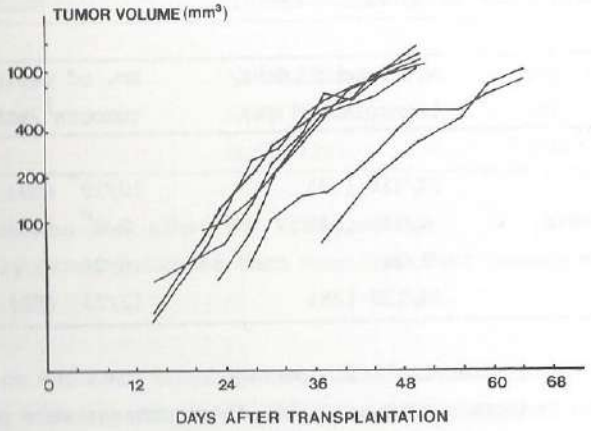

Fig. 2. Individual growth curves of seven xenografted tumours from the line HNX-KR in the second passage.

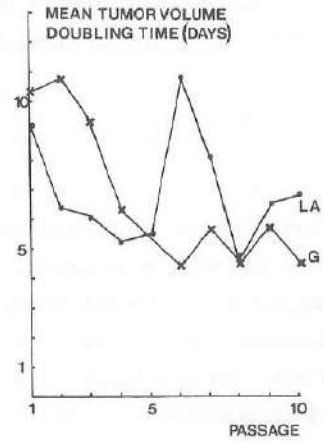

Fig. 3. Mean tumour-volume doubling time (100–200 mm^3) in subsequent passages for the tumour lines HNX-LA and -G. Data were obtained from at least seven tumours per passage. In the figure is HNX- omitted for the sake of clarity.

was too small. The mouse-grown tumour reached a volume of 100 mm^3 in 3–8 weeks and generally showed progressive growth, initially in an exponential manner. However, growth rates subsequently decreased (Fig. 2). In 4 tumour lines the tumour volume doubling time from 100 to 200 mm^3 decreased after two to four passages to a constant level of 4 to 6 days. In two lines the tumour volume doubling time varied between the individual passages from 5 to 11 days (Fig. 3).

Histological characteristics of the 12 tumour lines are summarized in Table 5. No major changes were observed after passaging in the nude mice. Most tumours were well circumscribed and surrounded by a connective tissue capsule, originating from the mouse. Mouse-grown tumours of all lines showed necrosis. The extent varied between tumour lines and increased according to tumour volume. In one tumour line HNX-W, only a thin rim of viable tumour tissue was left when the tumour reached a volume of 1000 mm^3. The remaining non-viable tissue consisted of keratin fibres and areas with scattered pyknotic cells. Invasion in the adjacent mouse skin was observed in one line. Such invasion was found in half of the cases, but there was no

invasion of muscles. No macroscopic or microscopic signs of metastasis to regional lymph nodes, lung, spleen, kidney or liver could be found in 300 tumour bearing animals.

DISCUSSION

From this study it has become clear that head and neck cancer can be grown in athymic nude mice. In 26% of the attempted transplantations tumour material started to grow subcutaneously in the mouse and serial passaging was possible in half of the cases. The initial take of 26% is lower than a mean value of 36%, obtained with various tumour types (11). This relatively low take rate might be attributed to the fact that with head and neck tumours a relatively high risk of contamination with bacteria and viruses is present. Contamination in a transplanted tumour might result in a stimulation of the residual immune system of the mouse, leading to an inhibition of tumour growth. Kyriazis et al. (13) showed that an infection with mouse hepatitis virus prevented tumour growth in a majority of nude mice.

That the remaining immune system can indeed play a role in inhibiting tumour growth has been shown by Kopper et al. (14) who established an increase in tumour take rate by damaging the macrophages with carrageenan. Habu et al. (15) found an increase in tumour take by blocking natural killer cells with an anti-serum. Immunosuppression, therefore, appears to be an attractive way to increase the take rate. Such an increase is required to reduce the number of transplantations .

Poorly differentiated head and neck squamous cell carcinomas tended to take better than moderately and well differentiated ones. This may be the result of a relatively higher proportion of clonogenic cells in poorly differentiated head and neck tumours, as indicated by the results of Mattox and Von Hoff (16). Using a double layer soft agar technique they found a tendency towards a higher cloning efficiency for poorly differentiated squamous cell carcinomas. A simpler explanation for the better take rate in poorly differentiated squamous cell carcinomas might be that these carcinomas contain more viable tumour cells than the more differentiated tumours, where parts of the tissue consist of non-dividing cells and keratin. In this regard Bastert et al. (17) found a higher take rate with connective

Table 5. Characteristics of tumour lines

Line	Histology*	Localization patients tumour	Invasion in mouse
HNX-P	well diff. scc.	Alveolar process	—
HNX-LA	poorly diff. scc.	piriform sinus	—
HNX-SG#	mod. diff. scc.	piriform sinus	—
HNX-PV	muco-epidermoid ca.	mandibular gland	—
HNX-G	well diff. scc.	skin of auricle	+
HNX-L#	well diff. scc.	lymph node	—
HNX-H	well diff. scc.	lymph node	—
HNX-O	mod. diff. scc.	palate	—
HNX-W	mod. diff. scc.	floor of mouth	—
HNX-TI	well diff. scc.	cheek mucosa	—
HNX-KR	poorly diff. scc.	floor of mouth	—
HNX-ML	poorly diff. scc.	hypopharynx	—

#These lines were lost after the fifth passage

*mod. diff. scc.: moderately differentiated squamous cell carcinomas

tissue-poor breast carcinomas than with connective tissue-rich carcinomas. Material from lymph node metastasis appears to take more readily than material from primary tumours. The higher take rate for material from metastasized tumours was also found by Fogh et al. (1) and Giovanella et al. (18) for various tumour types. This correlation was not found when breast tumours were used (17, 19). An explanation for the higher tendency of metastatic cells to grow in nude mice might be that the proliferation rate in these cells is higher than those in the primary tumour. Absence of contamination in the metastatic material might also be important.

In 4 of 6 tumour lines the take rate varied in subsequent passages. A similar finding is reported by Houghton and Taylor (7), who established 6 colorectal tumour lines in immune-suppressed mice. Mattern et al. (20) found a higher percentage of take with increasing passages for breast, lung and ovarium tumours, as observed for two tumour lines in this study.

In 4 of 6 lines an increase in growth rate, measured as the decrease of the tumour-volume doubling time from 100-200 mm^3, was seen during the first passages. Thereafter the tumour-volume doubling time varied between 4 and 6 days. This decrease in tumour volume doubling time was also found for breast carcinomas (19), transplanted in immune suppressed mice. Selection for faster growing cells or increase in the growth fraction might take place. An increase of cells in the S-phase was also found in the first passages when they were measured with flow-cytofluorometry (8) and ^{3}H-thymidine uptake (21). Fodstad et al. (22) did not observe a decrease in tumour volume doubling time of melanomas with increasing number of passages.

The histology of the xenografts was generally similar to that of the original tumour. In the literature changes in histology after transplantations are reported. Giovanella et al. (18) found an increase in differentiation in 25% of their material from various parts of the body. Dedifferentiation is also reported for lung (8) and breast carcinomas (10).

No signs of metastasis could be found in $\pm$ 300 tumour-bearing animals. In one tumour line invasion of the skin was observed in half of the transplanted tumours. The incidence of metastasis in the nude mice is low when using various tumour types (3, 23). The immune system of the nude mouse can play an inhibiting role in the development of metastasis. In 3 week old mice, which have low levels of natural killer cells, the incidence of metastasis is elevated (24).

It is possible with this model to obtain sufficient human head and neck tumour material outside the human body to enable fundamental and applied research on head and neck cancer. For other tumour types this model has already been shown to be of value in the field of tumour immunology. Since it can also contribute to the production of tumour specific antisera (25).

Most studies with this model have been concerned with applied research into the effect of various treatment modalities. Attention has been focused particularly on chemotherapy. Xenografts generally respond to agents that are clinically active (9,26,27). A study of bronchial carcinomas (28) has demonstrated that the xenografts reproduced the chemotherapeutic response pattern of their source tumours. Therefore, this model appears to be of value as a screening model for new chemotherapeutic agents. Such a screening model is also urgently needed for head and neck cancer. Further studies using this model are to be encouraged.

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REFERENCES

1. Fogh J, Orteo T, Tiso J, Sharkey FE, Fogh JM, Daniels WP. Twenty-three new human tumour lines established in nude mice. Expl Cell Biol 1980; 48: 229-239.
2. Giovanella BC, Stehlin JS, William LJ, Lee S, Shepard RC. Heterotransplantation of human cancers into nude mice. A model system for human cancer chemotherapy. Cancer 1978; 42: 2269-2281.
3. Shimosato Y, Kameya T, Nagai K and 4 others. Transplantation of human tumors in nude mice. J Natl Cancer Inst 1976; 56: 1251-1260.
4. Visveldt J, Povlsen CO, Rygaard J. Chromosome analyses of human tumours following heterotransplantation of the nude mouse mutant nude. Acta Pathol Microbiol Scand (A) 1972; 80: 169-176.
5. Pesce AJ, Bubel HC, DiPersio L, Michael JG. Human lactic dehydrogenase as a marker for human tumor cells grown in athymic mice. Cancer Res 1977; 37: 1998-2003.
6. Thomson DMP, Neville AM, Phelan K, Schwartz Scanzano R, Vandevoorde JP. Human cancer transplanted in nude mice retain the expression of their organ-specific neoantigens. Eur J Cancer Clin Oncol 1981; 17: 1191-1197.
7. Houghton JA, Taylor DM. Growth characteristics of human colorectal tumours during serial passage in immune-deprived mice. Br J Cancer 1978; 37: 213-223.
8. Mattern J, Haag D, Wayss K. Volm M. Growth kinetics of human lung tumours in nude mice. Expl Cell Biol 1981; 49: 34-40.
9. Povlsen CO, Jacobsen GK. Chemotherapy of human malignant melanoma transplanted in the nude mice. Cancer Res 1975; 35: 2790-2796.
10. Polvsen CO, Growth of human tumors in nude mice and chemotherapy studies in this model. In: Houchens DP, Ovejera AA, eds. Proceedings of the symposium on the use of athymic (nude) mice in cancer research. New York/Stuttgart: Fischer, 1978: 187-194.
11. List of human tumours transplanted to nude mice. In: Nomura T, Ohsawa N, Tamaoki N, Fujiwara K, eds. Proc 2nd Workshop on nude mice. Tokyo: University of Tokyo Press. 1977: 587-595.
12. Looney WB, Mayo AA, Allen PM, Morrow JY, Morris HP. A mathematical evaluation of tumour growth curves in rapid, intermediate and slow growing rat hepatomata. Br J Cancer 1973; 27: 341-344.
13. Kyriazis AP, DiPersio L, Michael JG, Pesce AJ. Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic mouse. Int J Cancer 1979; 23: 402-409.
14. Kopper L, van Hanh T, Lapis K, Timar J. Increased take rate of human tumour xenografts after carragheenan treatment. Eur J Cancer 1980; 16: 671-678.
15. Habu S, Fukui H, Shimamura K, and 4 others. In vivo effects of

anti-asialo GM1. Reduction of NK-activity and enhancement of transplanted tumor growth in nude mice. J Immunol 1981; 127: 34-38.
16. Mattox DE, Von Hoff DD. In vitro stem cell assay in head and neck squamous carcinoma. Am J Surg 1980; 140: 527-530.
17. Bastert G, Fortmeyer HP, Eichholz H, Michel RT, Huck R, Schmidt-Matthiessen H. Human breast cancer in thymusaplastic nude mice. In: Bastert GB, Fortmeyer MP, Schmidt-Matthiessen H, eds. Thymusaplastic nude mice and rats in clinical oncology. Stuttgart, New York: Fischer, 1981: 157-181.
18. Giovanella BC, Stehlin JS, Fogh J, Sharkey FE. Serial transplantation of human malignant tumors in nude mice and their use in experimental chemotherapy. In: Houchens DP, Ovejera AA, eds. Proceedings of the symposium of the use of athymic (nude) mice in cancer research. Stuttgart/New York: Fisher 1978: 163-179.
19. Bailey MJ, Ormerod MG, Imrie SF and 4 others. Comparative functional histopathology of human breast carcinoma xenografts. Br J Cancer 1981: 43; 125-134.
20. Mattern J, Haag D, Wayss K, Volm M. Significance of proliferation for the growth of xenografted human tumours in nude mice. Anticancer Res 1981: 1; 15-18.
21. Tropé C, Johansson JE, Alm P, Landberg T, Olsson H, Wennerberg J. Human malignant melanoma heterotransplanted to nude mice. Neoplasma 1981: 28; 585-591.
22. Fodstad O, Aass N, Pihl A. Assessment of tumour growth and of response to chemotherapy of human melanomas in athymic, nude mice. Br J Cancer 1980: (suppl IV) 41; 146-149.
23. Sharkey FE, Fogh J. Metastasis of human tumors in athymic nude mice. Int J Cancer 1979: 24; 733-738.
24. Hanna N, Fidler IJ. Expression of metastatic potential of allogenic and xenogenic neoplasms in young nude mice. Cancer Res 1981: 41; 438-444.
25. Grant AG, Duke D. Production of antibodies against antigens released from human pacreatic tumour xenografts. Br J Cancer 1981: 44; 388-395.
26. Kopper L, Steel GG. The therapeutic response of three human tumor lines maintained in immune-suppressed mice. Cancer Res 1975: 35; 2704-2713.
27. Osieka R, Houchens DP, Goldin A, Johnson RK. Chemotherapy of human colon cancer xenografts in athymic nude mice. Cancer 1977: 40; 2640-2650.
28. Shorthouse AJ, Smyth JF, Steel GG, Ellison M, Mills J, Peckham MJ. The human tumour xenograft - a valid model in experimental chemotherapy? Br J Surg 1980: 67; 715-722.

CHAPTER 3

ENHANCED SUCCESS RATE OF TRANSPLANTATION WITH HUMAN TUMOURS IN CYCLOPHOSPHAMIDE TREATED NUDE MICE

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ABSTRACT

Immunosuppression was investigated as a means to enhance the take rate of various human tumour types in nude BALB/c or B10.LP/Cpb mice. Cyclophosphamide (CY), known as an immunosuppressive agent was injected ip at a dose of 100 mg/kg 24 hr prior to subcutaneous implantation of tumour fragments obtained from established xenograft lines or from patients. In three out of eight ovarian adenocarcinoma tumour lines, with a take of 50% or less in untreated control animals, tumour take was significantly increased by CY treatment to values between 75 and 100%. No effect of CY treatment on tumour take was seen with squamous cell carcinomas of the head and neck region, lung and prostatic carcinomas. The growth rate of these xenografts was not affected by CY pretreatment. These results indicate that immunological mechanisms indeed can play a role in the inhibition of tumour growth in nude mice. CY pretreatment may enhance the success rate of transplantation, but this effect appears to be limited to certain tumours.

INTRODUCTION

Transplantation of human cancers in athymic nude mice for studies in the biological behavior or chemosensitivity of specific tumour types has been done with a variable rate of success. It is well established that a number of heterotransplanted tumours fail to grow in nude mice (1). For certain tumour types, such as prostatic and breast carcinomas, serially transplantable tumour lines could be established only occasionally (2). Non-T-cell-mediated immune mechanisms may be responsible for this inhibition of tumour growth, as suggested by the increased success rate for transplantation of tumours in irradiated (3) or antilymphocyte serum treated nude mice (4). It is possible that macrophages, which contribute to tumour resistance (5), play a critical part in graft rejection. Furthermore, there is growing evidence that natural killer (NK) cells play a role in the inhibition of tumour growth (6). This mechanism might be important in nude mice, since NK cell activity is higher in nude than in euthymic mice (7). These findings indicate that immunosuppression of nude mice might increase the take rate of human tumours.

In this investigation cyclophosphamide (CY) was chosen because of its immunosuppressive properties. Pretreatment with moderate to high doses of CY leads to an impairment of NK cell activity in normal (8,9) and nude (10) mice and this effect probably results in an augmentation of the incidence of spontaneous (11) and experimental (12-14) metastases in the lungs of normal mice. Also the function of macrophages, which are more active in nude than in normal mice (15), can be inhibited by CY treatment (16). CY does not have a direct cytotoxic effect on tumour cells, if administered 24 hr prior to tumour implantation. It has been shown that 4-hydroxycyclophosphamide, the only active metabolite of CY (17) is not detectable in the blood of nude mice 2 hr after ip injection of 100 mg/kg (18).

In this report we confirm that CY can depress NK cell activity in nude mice. For a variety of human tumour xenografts it was investigated whether the immunosuppressive properties of CY resulted in an enhancement of the tumour take rate.

MATERIALS AND METHODS

Tumours and animals.

Experiments were done in five different institutes, each with its own tumour types and animal strain. Characteristics of tumours and mice used in this study are listed in Table 1. The B10.LP/Cpb nu/nu mice were obtained from TNO, Zeist, the Netherlands. The BALB/c nu/nu mice were bred in the different institutes. Most experiments were done with established in vivo growing tumour lines, but also tumour material directly taken from patients was studied (Table 1). Except for the laboratories in Amsterdam where the mice are housed in cages covered with paper filters, the animals were kept in laminar flow cage racks. All experimental procedures were conducted in a laminar flowhood and all cages, bedding, food and water were sterilized before use. Serial passaging was done by transplanting tumour fragments sc on both sides of the animal, as described previously in more detail (19, 20). All animals were followed for three months for the development of tumours.

Tumour measurements.

Tumour size was measured once or twice a week depending on tumour

Table 1. Characteristics of mice and tumours

Investigator	Strain, sex, age (wks)	Tumour	Histology	Origin
B.J.M.B.	B10.LP/Cpb, ♀, 8-10	HNX-E, HNX-K, HNX-KR, HNX-SK, HNX-TI, HNX-W	squamous cell carcinoma	head and neck
		HNX-CM	melanoma	head and neck
M.M.N.	B10.LP/Cpb, ♀, 8-10	H134,* FKo, Ov.Gl, Ov.He, Ov.Pe, Ov.Ri(C), Ov.Sl	adenocarcinoma	ovary
T.S.	BALB/c, ♂, 4-8	XOV.30, XOV.31, XOV.39, #XOV.42#	adenocarcinoma	ovary
		XLO.2, XLO.4, #XLO.6	small cell ca.	lung
		XMEL.1	melanoma	skin
J.C.R.	BALB/c, ♂, 8-12	PC-3, [a]PC-3M, [a]PC-93[a]	adenocarcinoma	prostate gland
D.H.R.	BALB/c, ♂, 12-16	WD2845#	malignant fibrous histiocytoma	femur
		ME180,* NHIK*	squamous cell ca.	cervix

*These tumours were initially established from cell lines; in vivo passages 2-6 were tested.

#These tumours were directly taken from patients and tested in the first passage.

[a]With prostatic carcinomas, tumour induction was studied after sc injection of cells from suspension cultures (2.5 or 5.0 x 10^5 cells/injected site).

volume doubling time. Tumour volume was expressed as length x width x height x 1/2. A tumour take was scored if the tumour reached a volume of 50 mm^3. Latent period was defined as the time in days needed by the tumour to reach 50 mm^3. The time needed by a tumour to grow from 50-100 mm^3 is

defined as the volume doubling time. Each experimental group consisted of 4–7 animals, i.e. 8–14 tumours.

Immunosuppression.

The mice of each experimental group were injected ip 24 hr before tumour implantation. In a few experiments a dose of 300 mg/kg CY was used. CY (Endoxan-Asta, Asta Werke, Bielefeld, Federal Republic of Germany) was dissolved in distilled water immediately before use and administered in a volume of 0.2 ml/20 g body weight.

Measurement of NK cell activity.

The activity of NK cells was determined in a 4 hour ^{51}Cr release assay as described earlier (21). YAC-1 target cells were labeled with 200 μCi Na_2CrO_4 solution (50–400 μCi/mg ^{51}Cr, obtained from Amersham, Buckinghamshire, UK) and incubated with spleen cells from CY-treated or untreated nude mice. The percentage of specific activity was expressed as (release with effector cells – spontaneous release/total releasable radioactivity per sample) x 100.

Statistics.

Differences in tumour take were analyzed with the Fisher exact probability test (22). In other experiments the two-tailed Student's t-test was applied.

RESULTS

Treatment of BALB/c nu/nu mice with 100 mg/kg CY resulted in a significant inhibition of spleen NK activity 24 hours after injection ($p<0.05$). This depression of NK activity was about 50% with all effector to target (E:T) ratios used (Fig. 1). With the other strain of nu/nu mice (B10.LP/Cpb) a similar reduction of NK cell activity after CY treatment was found (data not shown).

The effect of CY 24 hours before tumour implantation on tumour take and growth rate was investigated with a large number of tumours of different histologic origin. With tumour types including lung carcinomas, squamous cell carcinomas and a melanoma of the head and neck there was little or no

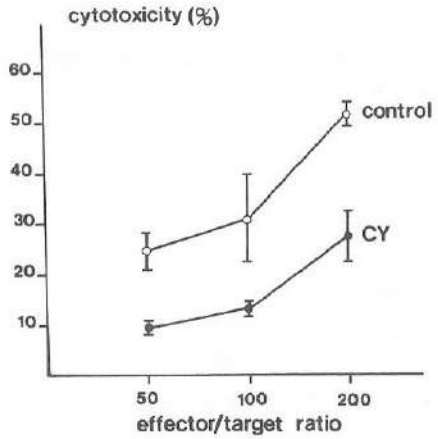

Fig. 1. Effect of CY treatment (100 mg/kg, -24 hr) on NK cell activity in BALB/c nude mice. Cytotoxicity was measured against 10^4 ^{51}Cr-labeled target cells at E:T indicated. Each group consisted of 4 different animals; cytoxicity was determined in quadruplicate. Values represent the mean $\pm$ s.d..

effect on the take rate (Fig. 2), which was reproducible in subsequent passages (Table 2). A significant ($p<0.05$) positive effect on tumour take was observed in 3 out of 8 ovarian adenocarcinoma lines in which the take rate in the control group was 50% or less (Fig. 3). For XOV.31, 7 out of 8 tumours grew in the CY group, and none of 8 tumours in the control mice. When the lines Ov.He and FKo were tested in two subsequent passages, the positive effect of CY on tumour take was reproduced (Table 2). This effect of CY in the ovarian tumour lines was stable for several passages (unpublished results). With the BALB/C strain in the Utrecht laboratory, a higher ip dose of 300 mg/kg was tolerated without signs of toxicity. Under these conditions a significant ($p<0.05$) increase in take rate of a malignant fibrous histiocytoma (in the first passage) was observed. Five out of ten tumours grew in the CY group, while none out of 12 in the control group showed growth.

The rate of tumour growth as reflected by the latent period and tumour doubling time was not influenced by CY pretreatment (data not shown). The results obtained with prostatic carcinoma cell lines in nude mice after sc injection suggest that pretreatment with CY may affect tumour growth in the

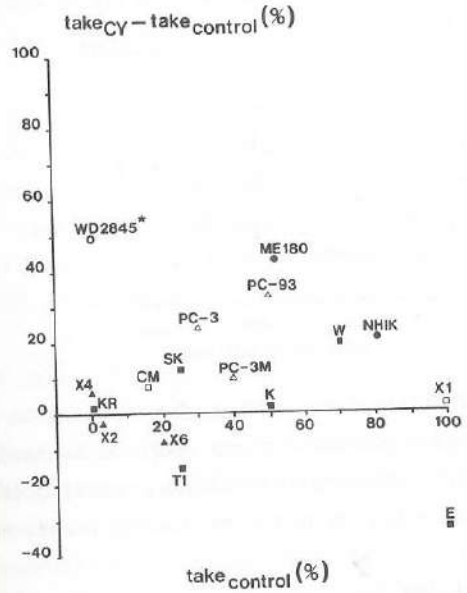

Fig. 2. Effect of CY pretreatment on tumor take of various types of tumours. With lung carcinomas (▲), melanomas (□), squamous cell carcinomas of the head and neck (■), and prostate carcinoma cell lines (△) 100 mg/kg CY was given. With the malignant fibrous histiocytoma (○) and squamous cell carcinomas of the cervix (●) 300 mg/kg CY was given. Omitted for the sake of clarity: "ME.", "LO." and "HNX-".

early growth phase. The latency time tended to be slightly shorter in the CY pretreatment group, but the differences were not statistically significant.

DISCUSSION

We have shown that pretreatment with CY can have a positive effect on the take of human tumours transplanted in nude mice. This effect seems to vary with tumour type. CY caused increased tumour take in three ovarian tumour lines and human sarcoma tissue in the first passage, but not in a number of other tumour types such as squamous cell carcinomas of the head

Table 2. Effect of CY on the take rate of tumours in two different passages

Tumour line	Passage	Take rate (%)	
		Control	CY
HNX-SK	5	2/8 (25)	3/8 (38)
	6	1/12 (9)	4/12 (25)
HNX-TI	7	2/8 (25)	1/10 (10)
	12	9/12 (75)	11/12 (92)
XLO.6	2	2/10 (20)	1/8 (13)
	3	2/10 (20)	1/10 (10)
Ov.He	13	3/12 (25)	11/12 (92)*
	14	3/10 (30)	10/10 (100)*
Ov.Ri(C)	7	8/12 (67)	10/12 (83)
	8	8/12 (67)	10/12 (83)
H134	2	3/8 (38)	3/12 (25)
	5	3/10 (30)	2/14 (14)
FKo	4	4/10 (40)	10/10 (100)*
	5	5/10 (50)	13/14 (93)*

Take rate is defined as the number of growing tumours/number of transplanted tumours. CY (100 mg/kg) was given ip 24 hr before implantation.

*Significant difference in take rate between CY and control group (Fisher exact probability test, $p<0.05$).

and neck region, lung and prostatic carcinomas. The results were obtained with two different strains of mice in five different laboratories.

The ineffectiveness of CY to influence the take of some tumours can be explained in two ways. First, the immunosuppression induced by CY might have been insufficient to allow the growth of tumours that are sensitive to immunologic defense mechanisms. Second, non-immunologic mechanisms that are not influenced by CY-treatment (e.g. availability of growth factors, the viability of the specimen at the time of transplantation) might be

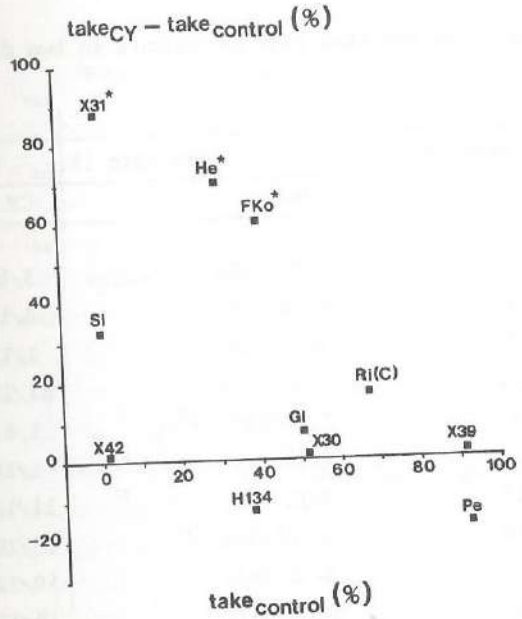

Fig. 3. Effect of CY pretreatment on the tumour take of ovarian adenocarcinomas in nude mice. Take rate is the number of growing tumors as a percentage of tumors transplanted. In the figure "OV." or "Ov" is omitted for the sake of clarity. Each group consisted of 8-14 tumors and when tested in more passages, only the take of the earlier passage is shown. *: statistically significant difference between CY and control group ($p<0.05$).

responsible for the prevention of growth of certain tumour types.

Since pretreatment with CY was effective in enhancing the take rate of some ovarian tumours, this implies that immune reaction mechanisms can indeed play a role in the inhibition of tumour growth in nude mice. This involvement of immunologic mechanisms in the rejection of tumours is in line with results reported by other investigators. Treatment of nude mice with antilymphocyte serum (4) or irradiation (3) enhanced the take rate of human cell lines. It is speculative, however, which non-T-cell mechanisms are responsible for tumour rejection in nude mice.

One component of the immune system that can be suppressed by CY is the NK cell, as shown in the present study and by other investigators (8-10).

Since CY pretreatment resulted in an enhanced take rate of some tumour lines, it is possible that in these lines NK cells are responsible for the inhibition of tumour growth. This finding is in agreement with the concept that NK cells play an important role in the immune surveillance against tumours (23, 24). The fact that in our experiments only some tumour lines showed an increased take after CY, can be explained by assuming that the population of tumours we have studied vary in NK cell sensitivity. To draw more definitive conclusions about this presumed variation in NK cell sensitivity one should assess whether a high in vitro NK sensitivity correlates with a low take rate in a particular tumour line. It was shown that tumour cells sensitive to NK cell killing in vitro are less tumourigenic in nude mice than NK cell resistant cells (25, 26). In contrast to these results, other investigators failed to confirm this association between in vitro cytolytic activity of NK cells and tumourigenicity (21, 27, 28). Thus, the susceptibility to NK cells, determined in vitro, is not a conclusive predictive indicator of tumourigenicity in nude mice. Additionally, it has been suggested that NK cells do not seem to exert any significant direct effect on tumour cells in vivo since the growth of some human and murine tumours was not increased in nude mice with low NK cell levels, compared to such growth in mice with high NK activity (29). This is in agreement with the results we have found in the tumour lines which did not show an enhanced take rate after CY treatment. In these lines NK cells may not play a role in tumour growth control.

The enhancement of tumour growth by CY might also be the result of suppression of immunologically active cell types other than NK cells. It is has been suggested that macrophages contribute to tumour resistance (5). Alveolar macrophage functions can be suppressed by CY (9) and it was recently reported that human monocytes could be functionally depressed by an active metabolite of CY (30). Thus, suppressive effects of CY on macrophages resulting in abrogation of the inhibition of tumour growth should be considered seriously. Also the humoral immune response can be depressed by CY (31). Nude mice are able to respond to human tumours with elevated antibody titers (32), but the significance of this phenomenon remains to be established.

It can be concluded that, though the mechanism of CY induced immunosuppression is not clearly known, pretreatment with this agent may enhance

tumour take. In tumour types with a low take rate in nude mice, CY pre-treatment should be employed in an attempt to achieve more efficient transplantation. It is not yet evident, why CY-mediated immunosuppression leads to an increased take of only certain tumours.

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REFERENCES

1. Steel GG, Peckham MJ. Human tumour xenografts: a critical appraisal. Br J Cancer 1980; 41, suppl. IV: 133-141.
2. Reid LCM, Shin S. Transplantation of heterologous endocrine tumor cells in nude mice. In: Fogh J, Giovanella BC, eds. The nude mouse in experimental and clinical research. New York: Academic Press, 1978; 313-351.
3. Prehn LM, Outzen, HC. Primary tumor immunity in nude mice. Int J Cancer 1977; 19:688-691.
4. Gershwin ME, Ikeda RM, Erickson K, Owens R. Enhancement of heterotransplanted human tumor graft survival in nude mice with antilymphocyte serum and in congenitally athymic-asplenic (lasat) mice. J Natl Cancer Inst 1978; 61:245-248.
5. Keller R. Promotion of tumor growth in vivo by antimacrophage agents. J Natl Cancer Inst 1976; 57: 1355-1361.
6. Herberman RB, Ortaldo JR. Natural killer cells: their role in defenses against disease. Science 1981; 214: 24-30.
7. Schultz RM. Factors limiting tumoricidal function of interferon-induced effector systems. Activated macrophages and natural killer cells. Cancer Immunol Immunother 1981; 10: 61-66.
8. Riccardi C, Barlozzari T, Santoni A, Herberman RB, Cesarini C. Transfer to cyclophosphamide-treated mice of natural killer (NK) cells and in vivo natural reactivity against tumors. J Immunol 1981; 126: 1284-1289.
9. Mantovani A, Luini W, Peri G, Vecchi A. Effect of chemotherapeutic agents on natural cell-mediated cytotoxicity in mice. J Natl Cancer Inst 1978; 61: 1255-1261.
10. Djeu JY, Heinbaugh JA, Vieira WD, Holden HT, Herberman RB. The effect of immunopharmacological agents on mouse natural cell-mediated cytotoxicity and on its augmentation by poly I:C. Immunopharmacology 1979; 1: 231-244.
11. Ruiter de J, Cramer SJ, Smink T, van Putten LM. The facilitation of tumor growth in the lung by cyclophosphamide in artificial and spontaneous metastasis models. Eur J Cancer 1979; 15: 1139-1149.
12. Ruiter de J, Smink T, van Putten LM. Studies on the enhancement by cyclophosphamide (NSC-26271) of artificial lung metastasis after labeled cell inoculation. Cancer Treat Rep 1976; 60: 465-470.
13. Vollmer TL, Conley FK. Effect of cyclophosphamide on survival of mice and incidence of metastatic tumors following intravenous and intracardial inoculation of tumor cells. Cancer Res 1984; 44: 3902-3906.
14. Hanna N, Fidler IJ. Role of natural killer cells in the destruction of circulating tumor emboli. J Natl Cancer Inst 1980; 65: 801-809.
15. Sharp AK, Colston MJ. The regulation of macrophage activity in congenitally athymic mice. Eur J Immunol 1984; 14: 102-105.
16. Hunninghake GW, Fauci AS. Immunological reactivity of the lung. IV. Effect of cyclophosphamide on alveolar macrophage cytotoxic effector function. Clin Exp Immunol 1977; 27: 555-559.
17. Brock N. Comparative pharmacologic study in vitro and in vivo with cyclophosphamide (NSC-26271), cyclophosphamide metabolites and plain nitrogen mustard compounds. Cancer Treat Rep 1976; 60:301-307.
18. Voelcker, G, Bastert, G, Fortmeyer HP, Haeglsperger R, Peter G, Hohorst H-J. Pharmacokinetics and therapeutic effects of "activated" cyclophosphamide (4-hydroxycyclophosphamide). In: Bastert GBA et al., eds. Thymus aplastic nude and rats in clinical oncology. Stuttgart/New York: Fischer, 1981: 255-261.
19. Braakhuis BJM, Sneeuwloper G, Snow GB. The potential of the nude mouse xenograft model for the study of head and neck cancer. Arch Otorhinolaryngol 1984; 239: 69-79.
20. Boven E, van der Vijgh WJF, Nauta MM, Schlüper HMM, Pinedo HM. Comparative activity and distribution studies of five platinum analogues in nude mice bearing human ovarian carcinoma xenografts. Cancer Res 1985; 45:86-90.
21. Romijn JC. Growth of tumor cells with different sensitivity for murine natural killer cells in young and adult nude mice. Expl Cell Biol 1985; 53:24-31
22. Sokal RR, Rohlf FJ. Biometry. San Francisco: Freeman, 1969, 595-598.
23. Hanna N, Burton RC. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastasis in vivo. J Immunol 1981; 127: 1754-1758.
24. Brooks CG, Flannery GR, Willmott N, Austin EB, Kenwrick S, Baldwin RW. Tumour cells in metastatic deposits with altered sensitivity to natural killer cells. Int J Cancer 1981; 28:191-198.
25. Kawase I, Urdal DL, Brooks, CG, Henney CS. Selective depletion of NK cell activity in vivo and its effect on the growth of NK-sensitive and NK resistant tumor cell variants. Int J Cancer 1982; 29:567-574.
26. Warner NL, Woodruff MFA, Burton RC. Inhibition of the growth of lymphoid tumours in syngeneic athymic (nude) mice. Int J Cancer 1977; 20: 146-155.
27. Klein AS, Plata F, Jackson MJ, Shin S. Cellular tumorigenicity in nude mice. Role of susceptibility to natural killer cells. Expl Cell Biol 1979; 47: 430-445.
28. Stanbridge EJ, Ceredig R. Growth-regulatory control of human cell hybrids in nude mice. Cancer Res 1981; 41: 573-580.
29. Fodstad O, Hansen CT, Cannon GB, Statham CN, Lichtenstein GR, Boyd MR. Lack of correlation between natural killer activity and tumor growth control in nude mice with different immune defects. Cancer Res 1984; 44:4403-4408.
30. Jason M, Andrews BS, Colvin M, Friou GJ. In vitro effects of 4-hydroperoxycyclophosphamide on the morphology and function of human peripheral blood mononuclear phagocyte cells (macrophages). Cancer Res 1984; 44: 3936-3941.

31. Kolb J-PB, Poupon M-FM, Lespinats GM, Sabolovic D, Loisillier F. Splenic modifications induced by cyclophosphamide in C3H/He, nude, and "B"- mice. J Immunol 1977; 118: 1595-1599.
32. Adelman DC, Miller RA, Kaplan HS. Humoral immune responses to human lymphoma cell heterotransplants in the central nervous system of athymic, nude mice. Int J Cancer 1980; 25: 467-473.

CHAPTER 4

CHEMOTHERAPY OF HUMAN HEAD AND NECK CANCER XENOGRAFTS WITH THREE CLINICALLY ACTIVE DRUGS: CIS-PLATINUM, BLEOMYCIN AND METHOTREXATE

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ABSTRACT

Human head and neck tumours were successfully transplanted in athymic nude mice. In 14 xenograft lines the effect of 1 to 3 clinically active agents could be tested. Maximum tolerated doses were given daily for 3-7 days. Growth delay was estimated in terms of the number of volume doubling times gained by the treatment. Cis-platinum and bleomycin appeared to be effective agents. In all 6 lines in which cis-platinum was examined, growth delay sometimes followed by complete regression was achieved. In 6/7 lines a response to bleomycin was observed. There was a wide variation in sensitivity to cis-platinum and bleomycin among the different lines. Methotrexate, effective in 40-60% of patients with head and neck cancer, showed essentially no activity. Methotrexate produced a minimal growth delay in 1/11 lines treated. Two of the patients from whom xenografts were obtained responded to methotrexate treatment. The observed lack of activity of methotrexate against these tumour xenografts indicates that this model has limitations in the screening of new anticancer agents.

INTRODUCTION

The nude mouse xenograft model seems promising for the evaluation of anticancer drugs. Xenografted human tumours generally respond to agents that are active in the clinic (1-4). For a few tumours it could be demonstrated that xenografts reproduced the patterns of chemotherapeutic response of their source tumours (5-7). Furthermore, Shorthouse et al. (8) demonstrated in 16 bronchial carcinomas established in immunosuppressed mice that the response of the xenografts and their donor tumours were similar.

Single agent chemotherapy is still commonly administered to patients with head and neck cancer, since no superior efficacy of a combination treatment has been proven (9). Therefore head and neck xenografts are well suited for a comparison of the response of xenografted tumours, with that of head and neck tumours generally in clinical practice and their source tumour in particular. An evaluation of the sensitivity of head and neck cancer xenografts to three drugs that are widely used in the clinic is described. Two individual comparisons of patient and xenograft responses to the same agent are reported.

MATERIALS AND METHODS

Animals and tumours

Female nude mice (B10.LP/Cpb, 8-10 weeks old) were obtained from the Centraal Proefdierenbedrijf TNO (Zeist, the Netherlands). The mice were maintained under conditions described elsewhere (10). Cages, bedding, food and acidified water were autoclaved before use. Only head and neck tumours from previously untreated patients were selected for implantation. Tumour material was dissected in slices measuring 3 x 3 x 1 mm and implanted s.c. in the lateral thoracic region on both sides of the animal. Tumours growing in nude mice were serially transplanted in a similar way. Tumour growth was measured biweekly using vernier calipers. Tumour volume was calculated as length x width x height x 0.5 (11). Twelve lines were established in this laboratory, of which 8 were reported in detail (10). Two lines (HNX-J and -V) were described by Lindenberger (12). Lactate dehydrogenase (LDH) isoenzyme analysis of the xenografts showed that > 80% of the LDH in the tumour was of human origin (13).

Chemotherapy

To date 14 xenograft lines were found to be suitable for chemotherapy (see Table 1). The patients from whom lines HNX-TI and HNX-W were derived received single agent chemotherapy (methotrexate) following biopsy of the tumour for implantation. Sensitivity was tested in early passages (2-9). Chemotherapy studies could not be done in each passage because of a varying pattern of tumour take. The intention was to include 8 tumours both in a control and treated group. Experiments with < 5 tumours in a group were excluded. Chemotherapy was started when the tumours reached 100 mm^3 (range 50-150 mm^3). The tumours were randomly divided into treatment and control groups. Since the growth rate varied between individual tumours, treatment was usually started on different days. The duration of treatment was limited to a maximum of 10 days. The intention was to study drug effects in terms of growth delay rather than cures. The agent was given daily till a maximum tolerated dose was reached, i.e. the maximum weight loss of the mice was 15%. For methotrexate 2 other schedules were used with injections every 4 and 7 days.

Table 1. Characteristics of xenograft lines.

Nomenclature	Histology	Site
HNX-B	moderately diff.	oropharynx
HNX-G	well diff.	skin
HNX-J	mderately diff.	lymph node, larynx
HNX-KB	well diff.	lymph node, unknown
HNX-KE	poorly diff.	larynx
HNX-KR	poorly diff.	oral cavity
HNX-LA	well diff.	lymph node, oral cavity
HNX-LP	moderately diff.	oral cavity
HNX-P	well diff.	oral cavity
HNX-PV	(mucoepidermoid ca.)	oral cavity
HNX-SG	moderately diff.	hypopharynx
HNX-TI	well diff.	oral cavity
HNX-V	poorly diff.	larynx
HNX-W	moderately diff.	oral cavity

All tumours are squamous cell carcinomas except HNX-PV.

Using these 2 schedules of methotrexate, LD_{10} doses were studied.

For the treatment and control groups the mean values of time needed for tumour to grow 2 and 4 times their initial volume were calculated. The mean values of the control and treated groups were compared with one-way analysis of variance, followed by the Student-Newman-Keuls test (14). Before these tests could be employed the data were checked for homoscedasticity and normality. Growth delay was defined, according to Kopper and Steel (2) as the difference between the mean values of the time needed by the treated and control tumours to grow form 100 to 200 mm^3, divided by the mean value of the time needed by the control tumours to grow from 100 to 200 mm^3. In the same way the growth delay for tumour size increase from 100 to 400 mm^3 (i.e. for 2 doubling times) was determined. Growth delay is thus expressed in terms of the number of volume doubling times gained by the treatment. This method of analysis makes it possible to compare lines that have

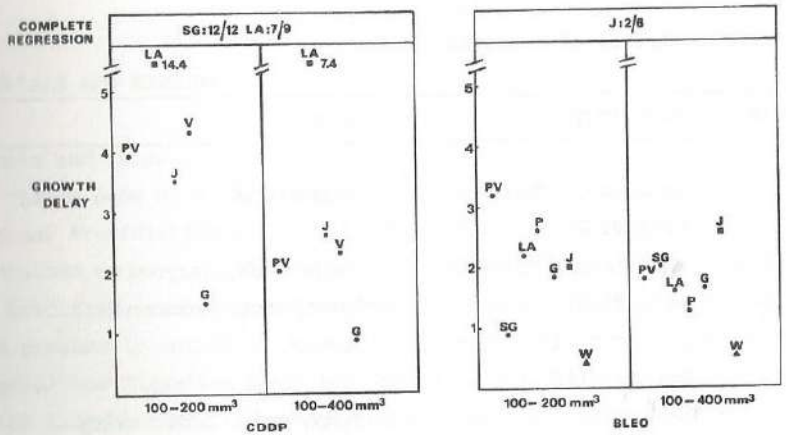

Fig. 1. Reaction of head and neck cancer xenografts to CDDP and BLEO. Growth delay is expressed in terms of the mean number of volume doubling times gained by the treatment. Difference between treated and control group from 100 to 200 and 400 mm^3, ●: significant ($p<0.05$). ▲:not significant ($p>0.05$) and ■: completely regressed tumours in the treated group, no significance tested. CDDP: 3 mg/kg daily for 3-5 days. BLEO: 15 mg/kg daily for 4-7 days. The number of completely regressed tumours divided by the numbers of treated tumours are shown in the upper panels.

different growth rates.

Drugs

Cis-platinum (CDDP, platinol, Bristol Meyers) was dissolved in distilled water immediately before i.p. injection. Bleomycin (BLEO, Lundbeck) was dissolved in distilled water and stored frozen. Injections were given s.c. in the back of the animal. Methotrexate (MTX, ledertrexate, Lederle) was dissolved in distilled water and kept at 4° C for a maximum of 14 days and injections were given i.p..

RESULTS

Three clinically active drugs were tested on 14 head and neck cancer

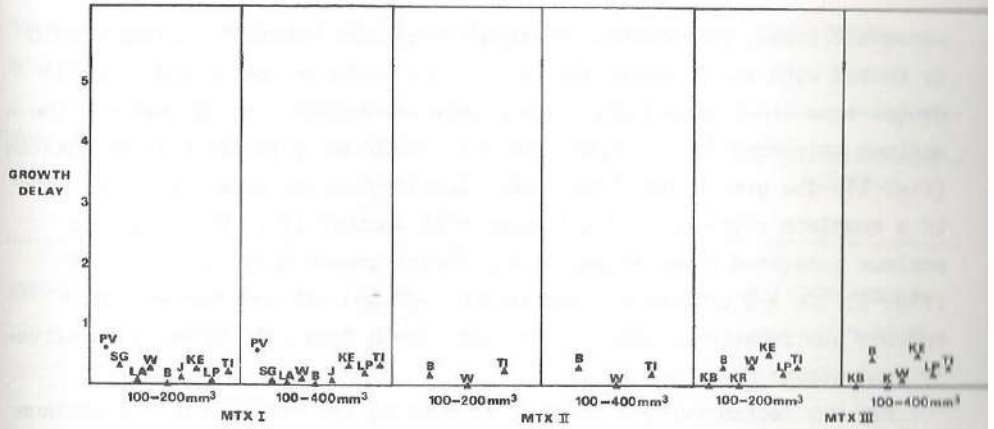

Fig. 2. Reaction of head and neck cancer xenografts to MTX: I Daily injections of 5 mg/kg for 5-7 days. II: Injections on days 1, 5 and 9. Mice with line W tumours received 50 and line B and line TI, 150 mg/ kg per injection. III: Injections on day 1 and 8, 250 mg/kg per injection, except lines KR and B which received 100 and 150 mg/kg respectively.

Table 2. Toxicity of methotrexate in nude mice with and without tumour.

	Dose/injection (mg/kg)	Injections on day	No. deaths/ total mice (%)
Tumour-bearing	250	1, 8	4/25[#] (16)
	5	1-5	8/35[*] (23)
Non-tumour-bearing	250	1, 8	1/7 (14)
	5	1-5	5/7 (71)

Groups of mice were injected i.p. The duration of the experiment was 40 days.

[#]Xenografts from 5 different lines.

[*]Xenografts from 6 different lines

xenograft lines, established in athymic nude mice. Three lines only could be tested with all 3 drugs. The other lines could not be tested with all drugs: some lines were lost, while others are still in early passage. The maximum tolerated dose of CDDP caused a growth delay in all 6 lines treated (Fig. 1). The growth delay for tumour doubling varied from 1.4 in one line to a complete regression of all tumours in another line. BLEO, using a maximum tolerated dose, showed a significant growth delay in 6/7 lines (Fig. 1). In 3/6 sensitive lines (HNX-G, -SG and -J) the regrowth of tumours was relatively late; for tumour growth from 100-400 mm^3 a relatively long growth delay was seen.

MTX was tested using a schedule of 5 mg/kg for 5-7 days till a maximum tolerated dose was reached. Only with one line (HNX -PV) was a small, but significant effect seen (Fig. 2). With the intention to mimic the situation in the clinic two high dose schedules were applied: (i) injections on days 1 and 8 and (ii) injections on days 1, 5 and 9. Toxicity studies with non tumour-bearing animals were performed to determine the LD_{10} dose. It was found that the number of deaths did not correlate with the dose. Sometimes a high dose was less toxic than a lower dose. For instance, 100 mg/kg MTX on day 1 and 8 appeared to be more toxic than 250 mg/kg. Because of this variability it was decided to give relatively high doses (Fig. 2). Another reason for the choice of these high doses was that the parallel toxicity study with a daily dose for 5 consecutive days showed that tumour-bearing animals appear to be less sensitive to MTX toxicity than non-tumour-bearing animals (Table 2). Using the schedule with injections on day 1 and 8 no difference in toxicity could be found between tumour- and non-tumourbearing animals.

With these high dose schedules MTX did not cause a growth delay in any xenograft line (Fig. 2). The effects of MTX on the tumours in two patients from whom xenografts were derived were known (Table 3). Tumours of patients corresponding to the lines HNX-TI and HNX-W regressed by 90 and 50% respectively after intra-arterial MTX treatment.

DISCUSSION

Xenografts of head and neck cancer can be grown successfully in athymic nude mice and used for chemotherapy studies. This study shows that, using

Table 3. Reactions to MTX of two head and neck tumours and their xenografts.

Tumour line	Schedule	Growth delay xenograft ($100-200mm^3$)	Patient Treatment	Patient Reaction
HNX-TI	5 mg/kg day 1-5	0.3	2 courses of MTX	90% tumour regression
	150 mg/kg day 1, 5, 9	0.2		
	250 mg/kg day 1, 8	0.3		
HNX-W	5 mg/kg day 1-5	0.3	4 courses of MTX	50% tumour regression
	50 mg/kg day 1, 5, 9	-0.2		
	250 mg/kg day 1, 8	0.3		

One course of MTX included a 24 hrs intra-arterial infusion followed by leucovorin-rescue. Xenografts were obtained from the patients prior to chemotherapy.

schedules with daily injections, CDDP was an effective agent; in all 6 xenograft lines a significant growth delay was found. BLEO was also effective; in only 1/7 lines was the growth delay insignificant. Clinical experiences with these agents have shown that BLEO and CDDP produce a partial or complete remission in 38 and 26% of patients, respectively (15). The reason for the superiority of BLEO and CDDP against the xenografted tumours

in comparison with the clinical data might be that only xenografts from sensitive patient tumours were tested. In this respect also BLEO and CDDP-insensitive head and neck cancer xenograft lines have been reported (16). In our data a better agreement with clinical findings could be obtained if those lines with a significant growth delay of less than two doubling times were considered insensitive.

It is interesting to note that MTX gave only a minimal response. Tested against 11 lines, only in one minimal growth delay was observed. Also bolus injections, which simulated the clinical situation did not result in growth delay. Partial or complete remission have been reported for MTX in 40% (17) to 60% (18) of treated patients. It is therefore unlikely that only MTX-resistant tumours were implanted into the nude mice.

It is worth noting that for two tumours the reaction of the xenografts to MTX did not correlate with the source tumours which were sensitive in the patient. This experience is contrary to all reported comparisons, showing similar resposes of the xenografts and the source tumours to the same treatment (5-8). This observed lack of correlation in the response to MTX might be attributed to a difference in pharmacokinetics of MTX between man and mouse. At least the route of administration is different. Also the duration of exposure of xenografts to MTX may be too short.

Resistance mechanisms potentially induced by xenografting may also account for the ineffectiveness of MTX in this model. The intracellular content of dihydrofolate reductase, the target enzyme to which MTX binds reversibly, may be increased or the rate of MTX uptake into the tumour cell may be decreased (19). Another possibility is a reversal of MTX toxicity by exogeneous purine and pyrimidine metabolites, released by a population of dying tumour cells. This cell loss could be higher in the xenografts than in the patients' tumour; a substantial part of the xenograft is often necrotic. This rescue mechanism can also be the basis of the protection against MTX toxicity by the presence of a tumour, as has been shown in the toxicity studies. With the low daily doses nucleic acid metabolites can play a role in protection against MTX toxicity since tumour (L1210)-bearing mice do not need purines to reverse MTX toxicity (20). Further studies are needed to elucidate the cause of MTX ineffectiveness.

In conclusion it is clear that the response to CDDP en BLEO varies for the xenograft lines, as has been shown by other authors using other tumours

and drugs (6, 21, 22). The lack of correlation between the xenograft and patient response to MTX appears to indicate that this model has certain limitations as a screening model for anticancer agents. It has yet to be established whether pharmacokinetic differences between man and mouse or resistance mechanisms in the xenograft itself are responsible for the lack of effect of MTX. It is also necessary that more direct comparisons between xenograft and patient response with the same agent are made.

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REFERENCES

1. Povlsen CO, Jacobsen GK. Chemotherapy of a human malignant melanoma transplanted in the nude mouse. Cancer Res 1975; 35: 2790-2796.
2. Kopper L, Steel GG. The therapeutic response of three human tumour lines maintained in immune-suppressed mice. Cancer Res 1975; 35: 2704-2713.
3. Osieka R, Houchens DP, Goldin A Johnson RK. Chemotherapy of human colon cancer xenografts in athymic nude mice. Cancer 1977; 40: 2640-2650.
4. Shorthouse AJ, Smyth JF, Steel GG, Peckham MJ. Experimental combination and single-agent chemotherapy in human lung-tumour xenografts. Br J Cancer 1982; 46: 35-44.
5. Giovanella BC, Stehlin JS, Fogh J, Sharkey FE. Serial transplantation of human malignant tumors in nude mice and their use in experimental chemotherapy. In: Houchens DP, Ovejera AA, eds. Proceedings of the Symposium on the use of athymic (nude) mice in cancer research. New York: Fischer, 1978; 163-179.
6. Nowak K, Peckham MJ, Steel GG. Variation in response of xenografts of colo-rectal carcinoma to chemotherapy. Br J Cancer 1978; 37: 576-584.
7. Fujita M, Hayata S, Taguchi T. Relationship on chemotherapy on human cancer xenografts in nude mice to clinical response in donor patient. J Surg Oncol 1980; 15: 211-219.
8. Shorthouse AJ, Smyth JF, Steel GG, Ellison M, Mills J, Peckham MJ. The human tumour xenograft-a valid model in experimental chemotherapy? Br J Surg 1980; 67: 715-722.
9. DeConti RC, Schoenfeld D. A randomized prospective comparison of intermittent methotrexate, methotrexate with leucovorin, and a methotrexate combination in head and neck cancer. Cancer 1981; 48: 1061-1072.

10. Braakhuis BJM, Sneeuwloper G, Snow GB. The potential of the nude mice xenograft model for the study of head and neck cancer. Arch Otolaryngol 1983; 239: 69–79.
11. Looney WB, Mayo AA, Allen PM, Morrow JY, Morris HP. A mathematical evaluation of tumour growth curves in rapid, intermidate and slow growing rat hepatomata. Br J Cancer 1973; 27: 341–344.
12. Lindenberger J. Aspects of xenografted tumors of the ear, nose and throat. Morphology, cell kinetics, growth behaviour and immmunology. In: Bastert GBA et al, eds. Thymusaplastic nude mice and rats in clinical oncology. Stuttgart: Fischer, 1981; 449–468.
13. Pesce AJ, Bubel HC, Dipersio L, Michael JG. Human lactic dehydrogenase as a marker for human tumor cells grown in athymic mice. Cancer Res 1977; 37: 1998–2003.
14. Sokal RR, Rohlf FJ. Biometry. San Francisco: Freeman & Co, 1969; 175–203.
15. Taylor SG. Head and Neck Cancer. In: Pinedo HM, ed. Cancer Chemotherapy: Annual 1, Amsterdam: Excerpta Medica 1979; 237–256.
16. Azar HA, Fernandez SB, Bros LM, Sullivan JL. Human tumor xenografts in athymic (nude) mice: chemotherapy trials in serially transplanted tumors. Ann Clin Lab Sci 1982; 12: 51–59.
17. Carter SK. The chemotherapy of head and neck cancer. Semin Oncol 1977; 4: 413–424.
18. Kirkwood JM, Canellos GP, Ervin TJ, Pitman SW, Weichselbaum R, Miller D. Increased therapeutic index using moderate dose methotrexate and leucovorin twice weekly vs. weekly high dose methotrexate–leucovorin in patients with advanced squamous carcinoma of the head and neck. Cancer 1981; 47: 2414–2421.
19. Chabner BA. Antimetabolites. In: Pinedo HM, ed. Cancer Chemotherapy. Amsterdam: Excerpta Medica 1980; 1–26.
20. Straw JA, Talbot DC, Taylor GA, Harrap KR. Some observations on the reversibility of methotrexate toxicity in normal proliferating tisues. J Natl Cancer Inst 1977; 58: 91–97.
21. Bailey MJ, Gazet JC, Smith LE, Steel GG. Chemotherapy of human breast-carcinoma xenografts. Br J Cancer 1980; 42: 530–536.
22. Houghton JA, Houghton PJ. On the mechanism of cytotoxicity of fluorinated pyrimidines in four human colon adenocarcinoma xenografts maintained in immune-deprived mice. Cancer 1980; 45: 1159–1167.

CHAPTER 5

LACK OF EFFECT OF METHOTREXATE ON HUMAN HEAD AND NECK TUMOURS TRANSPLANTED IN ATHYMIC NUDE MICE

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ABSTRACT

Human head and neck tumour tissues derived from 16 different patients were transplanted in athymic nude mice. Treatment of tumour-bearing animals with methotrexate had little or no effect on the doubling time of the xenografts. Included were three tumour lines derived from patients in whom methotrexate did demonstrate antitumour activity. These results are also in contrast to clinical experience with methotrexate, showing remissions in 50% of patients with head and neck cancer. It is unlikely that this lack of effect of methotrexate is attributed to a difference in drug pharmacokinetics between man and nude mouse, since a xenografted rat tumour was found to be sensitive. With regard to possible resistance mechanisms underlying methotrexate inactivity, we found no evidence of increased dihydrofolate reductase activity in the methotrexate-insensitive human tumour xenografts. It is possible that in this model a selection occurs favouring the outgrowth of a resistant subpopulation of tumour cells.

INTRODUCTION

Athymic nude mice have a T-cell immunodeficiency which allows the growth of human tumours in these animals. Regarding head and neck cancer about one-third of the tumours grow in nude mice. We observed a "take" rate of 26% (1), while Wennerberg et al. (2) and Lindenberger et al. (3) found a somewhat higher "take" rate of 35% and 36% respectively. In our studies (1) and those of Wennerberg et al (2), heterotransplanted tumours could be serially passaged in about half of the cases.

As the experience with cytostatic agents for head and neck cancers is limited, we have placed emphasis on the use of the nude mouse xenograft model for the screening of new drugs. To evaluate the potential of this model, clinically active drugs have been tested (4). Surprisingly, methotrexate (MTX) which is active in 50% of patients with head and neck tumours (5) showed only a minimal antitumour effect against a number of tumour lines. Further investigation to elucidate the factors responsible for the differential effect of MTX on head and neck tumours in nude mice and in patients could enhance the understanding of the model and may also reveal resistance mechanisms. This paper presents some further information on the

possible mechanisms responsible for the lack of effect of MTX.

MATERIALS AND METHODS

Animals and tumours

Female nude mice (B10.LP/Cpb, 8-10 weeks old) were obtained from the Centraal Proefdieren Bedrijf TNO (Zeist, the Netherlands). The conditions under which the mice were kept are reported elsewhere (1). Only head and neck tumours from previously untreated patients were selected for implantation. In addition, the rat rhabdomyosarcoma R1 was obtained from TNO (Rijswijk, the Netherlands) as a solid tumour and xenografted. This spontaneous tumour has been passaged both in vitro and in vivo and has been characterized earlier (6, 7). R1 has a high "take" rate in nude mice and a doubling time of 2.5 days. Tumour material was transplanted as described earlier (1). Also, the characteristics of most tumour lines were reported previously (1, 4, 8). Additional tumour lines included HNX-E, a moderately differentiated squamous cell carcinoma of the oral cavity, HNX-DU, an undifferentiated carcinoma of the hypopharynx, and HNX-GU, a moderately differentiated squamous cell carcinoma derived from a lymph node metastasis originating from an oropharynx tumour. The patients from whom the lines HNX-E, -TI and -W were derived, received chemotherapy with methotrexate after surgical biopsy of tumour tissue for implantation.

Chemotherapy

Sensitivity was examined during early passages (2-11). Tumour volume was calculated according to the formula 0.5 x (length x width x height). Animals bearing tumours with a volume between 50 and 150 mm^3 were randomly divided into treated and control groups (5-8 tumours/group). The methotrexate (MTX) dose chosen resulted in a weight loss of 10-25%. Deaths occurred in 0-25% of treated animals, varying with experiments. MTX (Ledertrexate, Lederle) was given i.p.. Cytostatic effect was expressed as tumour growth delay, according to Kopper and Steel (9). Growth delay was defined as the difference between the mean values of the time required by the tumours of treated and control animals to double their volume, divided by the mean value of the time required by the tumours of control mice to double their volume. Growth delay was thus expressed in terms of the fold

increase in volume doubling time gained by the treatment.

Dihydrofolate reductase assay

Dihydrofolate reductase (DHFR) activity was determined in tumours which were excised and frozen immediately in liquid nitrogen. Frozen tissue was pulverized with a microdismembrator (Braun, Melsungen, W. Germany), and the tissue powder was dissolved in Tris-HCl buffer (0.05 M, pH 7.5) in a proportion of 250 mg/ml buffer. The enzyme activity was assayed according to Bertino and Fischer (10).

RESULTS

The cytostatic effect of MTX in head and neck tumour lines was generally minimal (Fig. 1). In most of the 16 lines tested the effect was minimal or absent using different schedules, drug administered each day or every 4 or 7 days. A minor response was observed in two lines, HNX-DU and HNX-G. The rat R1 rhabdomyosarcoma transplanted in nude mice proved to be sensitive to MTX. Even complete regressions of this tumour were seen. The effect of MTX on 3 patients from whom tumour lines were established was known. The patients' tumours regressed by 25, 50 and 90% after intra-arterial MTX administration, while the xenografts (HNX-E, -W and -TI) showed only a minimal response. With the line HNX-W it was studied whether a microscopic effect could be seen after administration of 250 mg/kg MTX. The histology of the treated tumours did not differ from that of the untreated tumours on either day 1 or day 8 after drug administration.

Since resistance to MTX can develop by overproduction of DHFR, activity levels of this target enzyme were determined in the xenografted tumours. The enzyme activity levels for various tumour samples were all in the same order of magnitude, although the value for the MTX-sensitive rat R1 tumour exceeded those of the human squamous cell carcinomas (Table 1).

DISCUSSION

Human head and neck tumour tissue can be transplanted successfully into athymic nude mice. The use of this model to predict chemotherapy sensitivity in individual patients is limited, due to the low "take" rate

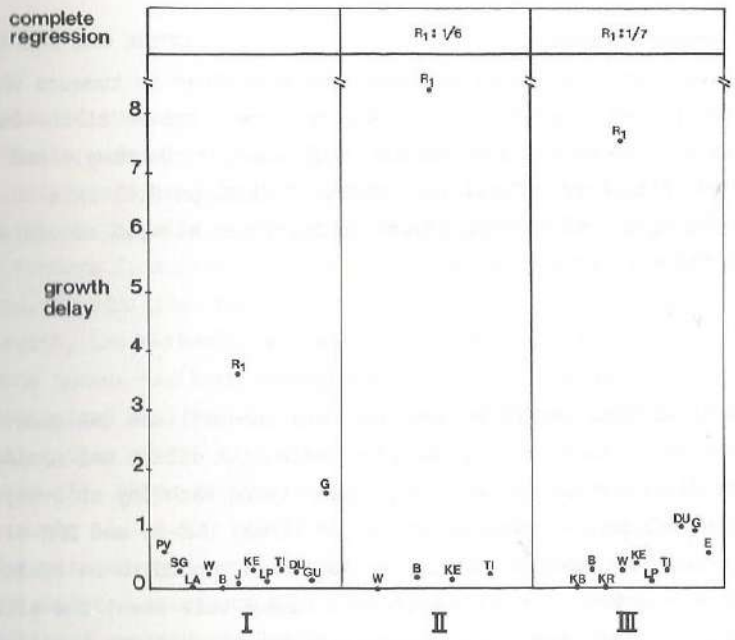

Fig. 1. Reaction of head and neck cancer xenograft lines to MTX. For a definition of growth delay: see material and methods. R1: rhabdomyosarcoma, growing in nude mice. As for the human tumour lines, the initial characters HNX are omitted for the sake of clarity. (I) Daily injections of 5 mg/kg for 5-7 days: line HNX-LA for 7, HNX-J for 6 and the remaining lines for 5 days. (II) Injections on days 1, 5 and 9. Line HNX-W received 50 mg/kg, the remaining 150 mg/kg per injection. (III) Injections on days 1 and 8, 250 mg/kg per injection, except lines HNX-KR, and -B which received 100 and 150 mg/kg respectively. The number of completely regressed tumours divided by the number of treated tumours is shown in the upper panel.

and the fact that long-term experiments are required. The model can be useful for the study of tumour cell kinetics (11) or in the screening of new drugs (12).

This study showed that MTX is minimally active in this model. Growth

Table 1. Dihydrofolate reductase activity in xenografted tumours

Tumour line	Passage number	DHFR activity#
HNX-TI	8	0.45
HNX-TI	14	0.63
HNX-G	10	0.73
HNX-G	11	—*
HNX-W	14	1.08
HNX-W	14	0.39
HNX-DU	9	0.18
HNX-DU	16	0.61
R1	9	2.72

Human head and neck tumour xenografts and a rat rhabdomyosarcoma (R1) were studied.

#nmol DHFR/mg protein/min

*-- not detectable

delay is much less than can be achieved with cis-platinum or bleomycin (4). The ineffectiveness of MTX in this model was unexpected, since 50% of patients with head and neck tumours respond to MTX (5). Moreover, in at least 2 cases the lack of response of the xenograft to MTX was consistent with demonstrated antitumour effect of MTX in these patients. The 2 patients' tumours clearly showed a response, while the effect in the xenografts was minimal, irrespective of the schedule used.

MTX acts as an antimetabolite, through it potent inhibition of the DHFR, the enzyme responsible for the conversion of folic acid to reduced folate factors (13). This leads to an inhibition of de novo synthesis of purine and thymine nucleotides, essential precursors of DNA and RNA. The biochemical mechanism of action of MTX involves effects on several complex metabolic pathways. Thus, there are various potential sites where the adverse effects of MTX may be either blocked or reversed, resulting in apparent drug resistance.

The lack of effect of MTX may be attributed to a difference in phar-

macokinetics or folate metabolism between man and nude mouse. In mice, MTX could be excreted more rapidly or folate co-factors could be more readily available to tissues. These are not supportable explanations, since the rat rhabdomyosarcoma, grown as a xenograft, was highly sensitive.

Known resistance mechanisms, potentially induced by xenografting, may also account for this lack of MTX effect. A plausible hypothesis is that tumour cells are rescued by exogenous nucleic acid precursors supplied by dying vicinal tumour cells. This concept of salvage pathway reversal of MTX cytocoxicity is supported by the finding that MTX is less toxic in tumour-bearing animals, than in non-tumour-bearing animals (4). It remains to be explained why the result is observed only with a low dose (5 mg/kg each day for 5 days) and not with a high-dose schedule (250 mg/kg twice a week). It is also possible that inherent differences between the tumour xenografts and the rat tumour exist which account for a differential capacity of re-utilization of circulating nucleobases and nucleosides. There is some evidence that head and neck tumours as well as normal tissues are rescued from MTX cytotoxicity with thymidine administration (14).

It is known from in vitro work that prolonged cultivation of cells in the presence of MTX can lead to drug resistance as a result of a 100-500 fold increase in the production of intracellular DHFR (15). Analysis of the DHFR activity in xenografted tumours specimens showed that the level of this enzyme was for all lines in the same order of magnitude as that of the rat rhabdomyosarcoma. The data are not consistent with a mechanism of resistance involving overproduction of DHFR in human head and neck xenografts. However, the emergence of DHFR with altered kinetic properties cannot be ruled out.

Another potential mechanism of resistance which has received considerable attention is the modification of drug transport across the cell membrane (16). This possibility has yet to be investigated in this model. A comparison of MTX and antifolates which do not require carrier-mediated transport may be quite useful in examining this problem.

It is believed that the intracellular formation of polyglutamate derivatives of MTX is important for enhanced cellular retention of the drug (17). MTX polyglutamates act as an intracellular depot of MTX and minimize the normally rapid transport of free drug out of the cell. Impaired formation of polyglutamates could lead to insufficient exposure of cells to

MTX and could affect MTX resistance.

It still remains to be established to what degree a more malignant population of cells is selected in the nude mouse model. By means of flow cytometry it was found that in the nude mouse model, selection of aneuploid cell populations occurred when head and neck tumours were transplanted in nude mice (18). We have noted that head and neck cancer xenografts have a higher clonogenicity in soft agar than tumours taken directly from the patients (19). It is conceivable that due to this selection process the population of MTX-sensitive cells is lost. Such a selection would indicate dissimilarities between xenografts and patients' tumours, and this can have new implications for the understanding and interpretation of chemosensitivity studies using this model.

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REFERENCES

1. Braakhuis BJM, Sneeuwloper G, Snow GB. The potential of the nude mouse xenograft model for the study of head and neck cancer. Arch Otolaryngol 1984; 239: 69-79.
2. Wennerberg J, Tropé C, Björklund A. Heterotransplantation of human head and neck tumours into nude mice. Acta Otolaryngol (Stockh) 1983; 95: 183-190.
3. Lindenberger J, Ganzer U, Fortmeyer HP. Die Heterotransplantation bösartiger Kopf- und Halsgeschwülste auf thymusaplastische nackte Mäuse. Arch Otorhinolaryngol 1978; 220: 117-128.
4. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Sneeuwloper G, Snow GB. Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cis-platinum, bleomycin and methotrexate. Br J Cancer 1983; 48: 711-716.
5. Hong WK, Bromer R. Chemotherapy in head and neck cancer. N Engl J Med 1983; 309: 75-79.
6. Reinhold HS. A Cell dispersion technique for use in quantitative transplantation studies with solid tumours. Eur J Cancer 1965; 1: 67-71.
7. Barendsen GW, Broerse JJ. Experimental radiotherapy of a rat rhabdomyosarcoma with 15 Me V neutrons and 300 kV X-rays. I. Effects of single exposures. Eur J Cancer 1969; 5: 373-391.
8. Lindenberger J. Aspects of xenografted tumors of the ear, nose and

throat. In: Bastert GBA et al, ed. Thymusaplastic nude mice and rats in clinical oncology. Stuttgart: Fischer, 1981, 449-467.
9. Kopper L, Steel GG. The therapeutic response of three human tumor lines maintained in immune-suppressed mice. Cance Res 1975; 35: 2704-2713.
10. Bertino JR, Fischer GA. VI. Techniques for the study of resistance to folic acid antagonists. Methods Med Res 1964; 10: 297-307.
11. Wennerberg J, Alm P, Biörklund A, Killander D, Långström E, Tropé C. Cell cycle perturbations in heterotransplanted squamous-cell carcinoma of the head and neck after mitomycin C and cisplatin treatment. Int J Cancer 1984; 33: 213-222.
12. Osieka R, Houchens DP, Goldin A, Johnson RK. Chemotherapy of human colon cancer xenografts in athymic nude mice. Cancer 1977; 40: 2640-2650.
13. Schornagel JH, McVie JH. The clinical pharmacology of methotrexate. Cancer Treat Rev 1983; 10: 53-75.
14. Schornagel JG, Leyva A, Pinedo HM. Methotrexate with thymidine protection or rescue in advanced head and neck cancer: a phase II study. Cancer Treat Rep 1984; 68: 543-545.
15. Diddens H, Niethammer D, Jackson RC. Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB 3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 1983; 43: 5286-5292.
16. Chabner BA. Antimetabolites. In: Pinedo HM, ed. Cancer Chemotherapy. Amsterdam: Excerpta Medica, 1980; 1-26.
17. Fry DW, Anderson LA, Borst M, Goldman ID. Analysis of the role of membrane transport and polyglumation of methotrexate in gut and the Ehrlich tumor in vivo as factors in drug sensitivity and selectivity. Cancer Res 1983; 43: 1087-1092.
18. Wennerberg J. Changes in growth pattern of human squamous-cell carcinomas of head and neck during serial passages in nude mice. Int J Cancer 1984; 33: 245-250.
19. Heinerman ECM, Braakhuis BJM, Boerrigter GH, Snow GB. Successful in vitro growth of human head and neck cancer after transplantation in nude mice. Arch Otolaryngol 1985; 241: 225-232.

CHAPTER 6

ACTIVITY OF CONVENTIONAL DRUGS IN HEAD AND NECK CANCER XENOGRAFTS

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ABSTRACT

Nude mice bearing human head and neck cancer xenografts were treated with five anticancer drugs active in patients with this type of tumour. Using maximum tolerated dose levels bleomycin appeared to be the best drug, with activity in 4/9 lines tested; a growth delay factor (GDF) of more than 2 was taken as the criterion of activity. 5-Fluorouracil and cyclophosphamide caused moderate responses (1<GDF<2) in respectively in 4/7 and 4/6 of the lines. Methotrexate and cisplatin were minimally or not active in the respectively 11 and 10 lines investigated. The activity of cisplatin appeared to be dose-dependent. In the past higher doses could be given, resulting in 5/6 responding lines. In four cases a direct retrospective comparison could be made between the two schedules: four lines did not respond to treatment with the present schedule, but were sensitive to treatment in the past, when the higher total dose was used. The activity of methotrexate is less than could be expected from clinical studies. The mechanism responsible for this lack of activity of this drug is discussed in relation to our recent results.

INTRODUCTION

Due to their immunodeficiency athymic nude mice allow the growth of human tumours. Regarding head and neck cancer we observed a 'take rate' in the first passage of 26% (1), which is in line with the results of other authors (2-4). Xenografted head and neck tumours can be passaged serially in about one half of the cases, that showed growth in the first passage (1, 4).

The nude mouse model could play an important role in the screening of new agents for head and neck cancer. First, however, it has to be established how chemotherapy results in this model correlate with the results in the clinic. This can be achieved by testing a number of conventional drugs in a panel of head and neck tumour lines. The results will give also information on the variation in sensitivity among the different lines. This will be important for the choice as to how many lines have to be used when the activity of a new drug is analysed. In this paper we describe the sensitivity of a number of head and neck tumour lines to five drugs that

are active in clinical practice (5).

MATERIALS AND METHODS

Animals and tumours.

Female nude mice (B10.LP/Cpb, 8-10 weeks old) were obtained from the Centraal Proefdieren Bedrijf TNO (Zeist, The Netherlands). The conditions under which the mice are kept are reported elsewhere (6). Tumour fragments were transplanted subcutaneously. The characteristics of the lines used have been described earlier (1, 6-8). With the exception of the line HNX-DU, which originated from an undifferentiated carcinoma, all tumours were derived from squamous cell carcinomas, varying in degree of differentiation and site of origin.

Chemotherapy.

Tumour volume was calculated according to the formula 0.5 x length x width x height. Animals bearing tumours with a volume between 50 and 150 mm^3 were randomly divided into treatment and control groups with 5-8 tumours per group. Drugs were given intraperitoneally at a maximum tolerated dose level (5-15% weight loss). Doses and schedules are summarized in Table 1. An antitumour effect was expressed as tumour growth delay (8). The growth delay factor (GDF) was defined as the difference between the mean values of the time required by tumours of treated and control animals to double their volume, divided by the mean value of the time required by the tumours of the control mice to double their volume.

Drugs

Bleomycin (Lundbeck) was dissolved in distilled water and stored frozen at -20 °C. Cisplatin (CDDP, Bristol Myers) was obtained as a solution and further diluted with distilled water. Methotrexate (MTX, Lederle) was dissolved in distilled water and kept at a maximum at 4 °C for a maximum of 14 days. Cyclophosphamide (Asta Werke) was dissolved in distilled water immediately before use. 5-Fluorouracil (Hoffmann-La Roche) was dissolved in distilled water and stored in the dark at room temperature.

Table 1. Schedule of conventional drugs in nude mice with head and neck cancer xenografts.

Drug	Dose (mg/kg)	Days of treatment
MTX	250	0, 7
MTX	5	0-4
CDDP	2	0-2
Bleomycin	15	0-2
5-Fluorouracil	25	0-3
Cyclophosphamide	100	0, 7

RESULTS

Five conventional agents that are active in the clinic in 20-40% of patients with head and neck cancer (5) were tested on human head and neck cancer xenografts (Table 2). Two criteria of activity were chosen; drugs that cause a GDF of more than 1 and less than 2, were defined as moderately active, while a GDF of more than 2 would indicate drugs that are active. With the schedules used (Table 1) bleomycin appeared to be the most active drug, with 4/9 responsive lines when the criterion of activity of a GDF > 2 is chosen. 5-Fluorouracil and cyclophosphamide were only moderately active in respectively 4/7 and 4/6 of the lines. Methotrexate (MTX) and CDDP are minimally or not active.

In our earlier studies a higher total dose was tolerated for CDDP and bleomycin than reported here, whereas the animal toxicity, expressed as weight loss, was similar. When the results of all our experiments with CDDP are summarized, it can be noted that a higher total dose of CDDP lead to a response in a higher proportion of lines tested (Table 3). In four lines this dose dependent response was also observed in a direct retrospective way. Treatment with the low total dose of 6 mg/kg always resulted in a GDF of less than 0.5, whereas higher total doses caused a GDF of more than 1 in all lines (Table 4). Interestingly with bleomycin such a strong dose response relationship was not observed. The overall sensitivity to this

Table 2. Efficacy of conventional drugs in human head and neck cancer xenografts.

Drug	GDF[a] > 1[#]	GDF[a] > 2[#]
MTX*	1/11 (9%)	0/11 (0%)
MTX**	1/10 (10%)	0/10 (0%)
CDDP	0/ 8 (0%)	0/ 8 (0%)
Bleomycin	6/ 9 (67%)	4/ 9 (44%)
5-Fluorouracil	4/ 7 (57%)	0/ 7 (0%)
Cyclophosphamide	4/ 6 (67%)	0/ 6 (0%)

All drugs were given at a maximum tolerated dose level as specified in Table 1.

[a]growth delay factor; see materials and methods.

[#]no. sensitive/ no. tested lines

*250 mg/kg q7dx2

**5 mg/kg qdx5

drug did not change considerably when we were forced to decrease the total dose.

We have tested MTX with a daily dose schedule in 10 lines and only in one line was a moderate response observed. The same applies for the weekly dose schedule.

DISCUSSION

In this paper we report the activity of five conventional drugs tested in nude mice bearing head and neck cancer xenografts. Bleomycin was the most active drug; 44% of the lines responded when the criterion of response of a GDF > 2 was chosen. Also in the clinic responses to this drug are seen in about 20 to 40% of patients (5, 9). This drug did not show any substantial activity in 7/8 of the murine tumour models used at the NCI (10), which shows that the value of these murine tumours in the selection of anticancer agents is questionable.

Table 3. Response to CDDP and bleomycin in head and neck cancer

Drug	Total dose (mg/kg)	Sensitivity criterium	No. sensitive/ tested lines
CDDP	9–15	GDF[#] > 1	12/13 (92%)
	9–15	GDF > 2	6/13 (46%)
	6	GDF > 1	0/ 8 (0%)
Bleomycin	75–90	GDF > 1	5/ 6 (83%)
	75–90	GDF > 2	4/ 6 (67%)
	45–60	GDF > 1	6/ 9 (67%)
	45–60	GDF > 2	4/ 9 (44%)

CDDP was given daily. For the total dose of 6 mg/kg daily injections of 2 mg/kg and for the higher doses 3 mg/kg were given.

Bleomycin: 15 mg/kg per injection per day.

[#]growth delay factor: see materials and methods

Table 4. Effect dose CDDP on response of xenografts.

Line	Early passage			Late passage		
	no.	total dose[#]	GDF	no.	total dose[#]	GDF
HNX-FR	5	9	1.0	8	6	0.1
HNX-E	6	9	1.4	9	6	0.2
HNX-KE	6	9	1.5	10	6	0.5
HNX-G	6	12	1.4	11*	6	0.1

[#]in mg/kg for details, see Table 3.

*after freezing and thawing

Surprisingly CDDP did not elicit any response in the 8 tumour lines. This poor activity of CDDP in head and neck tumour lines is not in agreement with our earlier results (8). An essential difference with this earlier study is however, that at this moment we are forced to use lower total doses of CDDP. The same strain of mice is used, but due to an an unexplained cause the mice tolerate less. It is very likely that the poor activity to CDDP is mainly attributable to the fact that the mice have to be treated with less drug. This dose dependent responsiveness is stressed by the data in Table 4, which show that with the dose the response decreased in four lines. Also Wennerberg et al. (11) could demonstrate a dose response relationship with CDDP in human head and neck cancer xenografts. Also for bleomycin a lower total dose had to be given in the course of time, but this did not result in a strong decrease of activity (Table 3). The phenomenon that the dose dependent responsiveness varies with drug can have implications for the screening of new drugs. In our case CDDP would only have been detected as an attractive drug in our earlier studies in which the mice could tolerate more of the drug. This phenomenon can also happen when 2 strains of mice are used with a different CDDP tolerance. It is known that toxicity for a variety of anticancer drugs varies with the strain of mice (12, 13). Thus, choice of the strain of mice may influence the response to CDDP and perhaps also the response to new drugs.

As has been reported previously (8), in two cases a direct comparison could be made between the responses of the patient tumours and the tumour lines derived from these tumours. The patient tumours regressed after treatment with MTX whereas the corresponding xenografts did not respond to treatment, irrespective of the schedule used (7, 8). In our opinion the study of the underlying mechanisms of this insensitivity is very important. What should be determined is the effect of the host on the tumours when they are grafted. This study might also reveal resistance mechanisms, additional to the ones known from normal, euthymic mice and in vitro studies.

MTX is an inhibitor of dihydrofolate reductase (14). This enzyme is responsible for the conversion of dihydrofolate to reduced folate factors. This leads to an inhibition of the synthesis of purine and pyrimidine nucleotides, precursors of DNA and RNA. Also one or more glutamates can

chemically bind to MTX to form MTX-polyglutamates, which are retained longer in the cell (15).

To date we have investigated several possible mechanisms in order to explain this insensitivity to MTX in head and neck tumour lines. It is unlikely that the lack of effect of MTX in head and neck cancer xenografts is attributed to a difference in pharmacokinetics between man and nude mice, since a xenografted rat tumour was found to be sensitive (7). Further, we did not find evidence for an increased dihydrofolate reductase activity in the MTX-insensitive xenografts (7). Also there is evidence that impaired drug transport does not play an important role in this insensitivity, since MTX is taken up by the cells measured as an inhibition of DNA-synthesis in insensitive tumour lines after MTX administration (unpublished results). A likely possibility is now, that the retention of MTX is too short, perhaps caused by an absence of synthesis of polyglutamate forms of MTX.

In conclusion, our results show, that with some drugs problems arise in the nude mouse xenograft model. The activity of MTX is less than could have been expected from clinical studies and it is worth noting that this also applies to another anti-metabolite, 5-fluorouracil, when tested on gastrointestinal tumour xenografts (16). For CDDP a dose dependent effect was observed, which can have implications for the choice of strain of mice. Due to the limited number of lines tested, it is at this moment too early to draw conclusions about a correlation between the xenograft model and the clinical situation.

REFERENCES

1. Braakhuis BJM, Sneeuwloper G, Snow GB. The potential of the nude mouse xenograft model for the study of head and neck cancer. Arch Otorhinolaryngol 1984; 239: 69-79.
2. Lindenberger J, Ganzer U, Fortmeyer HP. Die Heterotransplantation bösartiger Kopf- und Halsgeschwülste auf thymusaplastische nackte Mäuse. Arch Otorhinolaryngol 1978; 220: 117-128.
3. Wennerberg J, Tropé C, Biörklund A. Heterotransplantation of human head and neck tumours into nude mice. Acta Otolaryngol 1983; 95: 183-190.
4. Hill JH, Plant RL, Harris DM, Grossweiner LI, Rok B, Seter AJ. The nude mouse xenograft system: a model for photodetection and photodynamic therapy in head and neck squamous cell carcinoma. Am J Otolaryngol 1986; 7: 17-27.
5. Hong WK, Bromer R. Chemotherapy in head and neck cancer. N Engl J Med

1983; 308: 75-79.
6. Braakhuis BJM, Nauta MM, Romijn JC, Rutgers DH, Smink T. Enhanced success rate of transplantation with human tumors in cyclophosphamide-treated nude mice. J Natl Cancer Inst 1986; 76: 241-245.
7. Braakhuis BJM, Leyva A, Schoevers EJ, Boerrigter GH, Schornagel JH, Snow GB. Lack of effect of methotrexate on human head and neck tumours transplanted in athymic nude mice. Acta Otolaryngol 1985; 99: 208-213.
8. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Sneeuwloper G, Snow GB. Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cis-platinum, bleomycin and methotrexate. Br J Cancer 1983; 48: 711-716.
9. Carter SK. The chemotherapy of head and neck cancer. Semin Oncol 1977; 4: 413-424.
10. Staquet MJ, Byar DP, Green SB, Rozencweig M. Clinical predictivity of transplatable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy. Cancer Treat Rep 1983; 67: 753-765.
11. Wennerberg J, Alm P, Biörklund A, Killander D, Långström E, Tropé C. Cell cycle perturbations in heterotransplanted squamous cell carcinoma of the head and neck after mitomycin c and cisplatin treatment. Int J Cancer 1984; 33: 213-222.
12. Houchens DP, Johnson RK, Gaston MR, Goldin A. Toxicity of cancer chemotherapeutic agents in athymic (nude) mice. Cancer Treat Rep 1977; 61: 103-104.
13. Freirich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. Cancer Chemother Rep 1966; 50: 219-245.
14. Schornagel JH, McVie JH. The clinical pharmacology of methotrexate. Cancer Treat Rev 1983; 10: 53-75.
15. Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA. Synthesis, retention and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 1982; 70: 351-360.
16. Fiebig HH, Schuchhardt C, Henss H, Fiedler L, Löhr GW. Comparison of tumor response in nude mice and in the patients. Behring Inst Mitt 1984; 74: 343-352.

CHAPTER 7

ACTIVITY OF SIX NEW DRUGS IN HEAD AND NECK CANCER XENOGRAFTS

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ABSTRACT

The nude mouse xenograft model was used to test six new agents for activity against head and neck cancer. Animals were treated intraperitoneally at maximum tolerated doses. 5-Aza-2'-deoxycytidine (5-aza-dCyd) a nucleoside analog, was active in one, modestly active in another and inactive in three tumour lines. The activity of 5-aza-dCyd was dose-schedule dependent; this result emphasizes the fact that new drugs ought to be tested with at least two schedules. Trimetrexate, a new folate antagonist, was tested in three tumour lines insensitive to methotrexate, and was shown to be inactive. The activity of 5'-deoxy-5-fluorouridine (5'dFUR), a prodrug of 5-fluorouracil (5-FU) was compared with that of 5-FU. In one line a better antitumour effect was seen with 5'dFUR than with 5-FU. In another line, insensitive to 5-FU, only a minimal response to 5'dFUR was detected. Three platinum analogs, TNO-6, JM-8 and JM-40 were tested in cisplatin insensitive lines. No superior effect of the analogs as compared to cisplatin was observed. When tested in a single head and neck tumor line 5'dFUR and 5-aza-dCyd have a better antitumour effect than conventional drugs; this makes these drugs of potential value for patients with head and neck cancer.

INTRODUCTION

There is an urgent need for new agents in the chemotherapeutic treatment of patients with head and neck cancer. With the conventional drugs remissions can be obtained in 20-40% of patients but usually survival time is not or only minimally increased (1).

The nude mouse model seems suitable for the evaluation of new anticancer drugs. Xenografted tumours respond to agents that are active in the clinic (2). At this moment it is too early to state that the results of the model always correlate with those obtained with patients in various tumour types. For head and neck cancer more results on the testing of conventional drugs in various lines should be evaluated (3).

Based on the results with the model so far and the urgent need for new drugs for head and neck cancer, we decided to test some new drugs in head and neck cancer xenografts. Our program was limited to the testing of drugs

Table 1. Characteristics of tumour lines

Line	Histology[#]	Site	Tumour volume doubling time[*]
HNX-DU	undiff. ca.	hypopharynx	4
HNX-E	mod. diff. scc.	oral cavity	11
HNX-FA	mod. diff. scc.	hypopharynx	9
HNX-FR	mod. diff. scc.	hypopharynx	9
HNX-G	well diff. scc.	skin	10
HNX-Hep-2[**]	poorly diff. scc.	larynx	8
HNX-KE	poorly diff. scc.	larynx	6
HNX-W	mod. diff. scc.	oral cavity	9

[#]mod. diff. scc.: moderately differentiated squamous cell carcinoma.
[*]as expressed in days
[**]established from a celline by subcutanous injection of $3x10^6$ cells.

that were studied in phase I trials. We argued that our results might help in the selection of compounds for subsequent testing in head and neck cancer phase II clinical trials.

MATERIALS AND METHODS.

Animals and tumours.

Experiments were carried out in a similar way as described previously (3). Characteristics of tumour lines are listed in Table 1. Antitumour effect was expressed as a growth delay factor (GDF), as reported previously (3).

Drugs.

Cisplatin (CDDP, platinol), JM-40, TNO-6 (spiroplatin) and JM-8 (carboplatin) were supplied by Bristol Myers International, New York, USA. 5-Fluorouracil (5-FU) and 5'-deoxy-5-fluorouridine (5'dFUR, doxifluridine) were donated by Hoffmann-La Roche, Mijdrecht, The Netherlands, 5-aza-2'-deoxycytidine (5-aza-dCyd) by Pharmachemie, Haarlem, The Netherlands and trimetrexate (TMQ) by Warner Lambert, Ann Arbor, USA. Methotrexate (MTX) was obtained from Lederle, Etten-Leur, The Netherlands. All drugs were dissolved as indicated by the manufacturers and injected ip at maximum tolerated dose levels (5-15% weight loss as compared to the untreated controls).

RESULTS

The activity of three new antimetabolites was analysed in five head and neck tumour lines and if possible compared with related conventional drugs.

5-Aza-dCyd had a substantial antitumour effect in one tumour line, HNX-DU, derived from an undifferentiated carcinoma of the hypopharynx. With a schedule of 2 mg/kg on day 0, 4 and 8 a GDF of 3.6 was seen and one out of six tumours regressed completely after treatment (Table 2). A minimal effect was noted, when the drug was given as a low dose daily for a longer period of time. When compared with other drugs it appeared that aza-dCyd and CDDP (given weekly) were the best agents in this line (Fig. 1). Aza-dCyd was also tested in four other tumour lines; in one line a moderate response was observed and in three lines the drug was inactive (Table 2).

In one line, HNX-KE, a poorly differentiated squamous cell carcinoma of the larynx, treatment with 5'dFUR, a structural analog of 5-FU, resulted in a better response than with 5-FU, with GDF-values of respectively 2.1 and 0.8 (table 3). When compared with conventional drugs 5'dFUR appeared to be the best agent in this tumour line (Fig. 2). In another, 5-FU insensitive line, the therapeutic effect of 5-FU could not be improved.

TMQ, a new lipophilic antifolate drug was tested in three MTX-insensitive lines; no effect was observed in these lines, when 50 mg/kg was given for four days (Table 3).

Three platinum analogs were tested as well and compared with CDDP. Administered in doses with comparable toxicity, no superior effect of any

Table 2. Activity of 5-aza-2'-deoxycytidine in head and neck cancer xenografts.

Line	Dose (mg/kg)	Injections on day	GDF*	Regression#
HNX-DU	2	0, 7	2.2	0/7
HNX-DU	2	0, 4, 8	3.6	1/6
HNX-DU	0.25	0-4, 7-11	0.2	0/7
HNX-DU	0.1	0-4##	0.2	0/4
HNX-Hep-2	2	0, 4, 8	0.5	0/9
HNX-KE	2	0, 4, 8	0.1	0/5
HNX-KE	0.1	0-4##	0.0	0/7
HNX-FA	2	0, 4	0.9	0/7
HNX-FR	2	0, 4	0.5	0/6

*growth delay factor; see: materials and methods of reference 3. Due to the fact that sometimes tumors regressed completely median values of tumour doubling times were used for the computation of GDF.

#no. regressed/no. treated tumours.

##three injections per day with a 4 hour interval for five days

of the analogs was observed in five CDDP-insensitive lines (Table 4).

DISCUSSION

New drugs were tested in human head and neck tumour lines and where possible, the activity was compared with conventional agents. 5-Aza-dCyd is an active antleukaemic agent in animal models (4) and children (5). The activity of the drug may be related to a differentiating inducing effect, probably caused by the hypomethylation of DNA (6). In the mechanism of action the formation of DNA strand breaks may play a role (7). This drug is unergoing clinical evaluation now in Europe in phase I and II trials in patients with solid tumours and leukaemias. In one head and neck tumour line it was found that 5-aza-dCyd was more active than the most conven-

Table 3. Activity of conventional and new antimetabolites in head and neck cancer xenografts.

Line	5-FU	5'dFUR	MTX	TMQ
HNX-DU	0.4	0.4	0.4	0.4
HNX-KE	0.8	2.1	0.3	0.6
HNX-W	n.t.	n.t.	0.1	0.0

Activity was expressed as growth delay factor (GDF), see: materials and methods of reference 3.
Drugs were administered i.p. in the following doses and schedules: 5-FU, 100 mg/kg, q7dx2; 5'dFUR, 1000 mg/kg, q7dx2; MTX, 5 mg/kg qdx5 and TMQ, 50 mg/kg qdx4.
n.t.: not tested

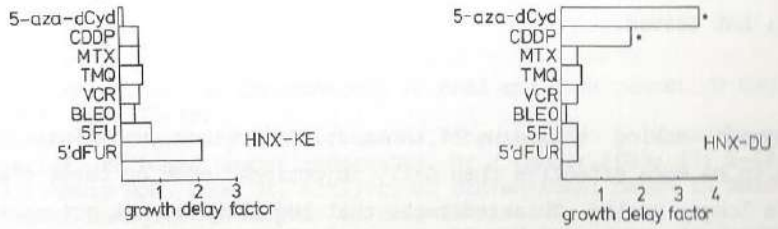

Fig. 1. Activity of conventional and new drugs in the head and neck tumour line HNX-DU and HNX-KE.
5-aza-dCyd: 5-aza-2'-deoxycytidine, 2 mg/kg, q4dx3; CDDP: cis-platin, 4 mg/kg, q7dx2; MTX: methotrexate, 250 mg/kg, q7dx2; TMQ: trimetrexate, 50 mg/kg, qdx4; VCR: vincristine, 1 mg/kg, qdx2; 5-FU: 5-fluorouracil, 50 mg/kg, q7dx2; 5'dFUR: 5'-deoxy-5-fluorouridine, 1000 mg/kg, q7dx2.
*1/6 tumours regressed completely

Table 4. Activity of platinum-analogs in head and neck cancer xenografts.

Line	CDDP	TNO-6	JM-8	JM-40
HNX-DU	0.4	0.0	1.5	0.2
HNX-DU	1.8*		0.2	
HNX-E	0.2	0.3	n.t.	n.t
HNX-FR	0.1	n.t	n.t	0.6
HNX-G	0.1	n.t.	0.1	0.8
HNX-KE	0.5	n.t.	0.8	n.t.

Activity was expressed as growth delay factor (GDF), see materials and methods of reference 3. Due to the fact that sometimes tumours regressed completely, median values of tumour volume doubling times were used for the computation of GDF.
Drugs were administered i.p. in the following doses and schedules:
CDDP, 2 mg/kg, qdx3; TNO-6, 1 mg/kg, qdx3; JM-8, 20 mg/kg, qdx3; JM-40, 10 mg/kg, qdx3.
*CDDP, 4 mg/kg, q7dx2, 1/6 of the treated tumours regressed.
n.t.: not tested.

tional agents, making this drug of interest. Injections every four days appeared to be more effective than daily injections, once or three times a day for a longer period. This indicates that the drug may not act as a metabolic inhibitor, since it can be assumed that this type of drug need a long exposure time to effect synthesis of DNA (8). Therefore, a DNA damaging or differentiation inducing effect cannot be ruled out. It is more likely that the analogue alters DNA-function, when incorperated into DNA. Also, this schedule dependent effect of 5-aza-dCyd makes clear that ,if possible, new drugs should be tested with at least two schedules to establish the activity of a drug.

Furthermore, our results show that in one head and neck tumour line 5'dFUR can be a better drug than 5-FU. The reason for this is not completely understood. The activity of pyrimidine nucleoside phosphorylase, an enzyme needed for the conversion of 5'dFUR to 5-FU might be increased in

tumour tissue. Also the mechanisms of action (9) or the pharmacokinetics may vary between the two drugs.

TMQ is a new lipophilic antifolate drug active in cell lines with a defective MTX transport (10). It does not need the high affinity carrier to enter a cell and it cannot be polyglutamated. The drug was inactive in 3 MTX-insensitive head and neck tumour lines with a schedule that proved to be active in L1210 a murine lymphoma in vivo (J.R. Bertino, personal communication). These results indicate that the MTX-insensitivity in these tumour lines is not likely to be caused by an impaired transport mechanism as discussed previously (3).

Our results with platinum analogues showed that none of the analogues had a superior activity as compared to CDDP. These results are in agreement with those of Boven et al. (11), who found cross-resistance to CDDP with the same analogs using human ovarian tumour lines.

In conclusion, our results show that 5-aza-dCyd and 5'dFUR are drugs with potential value for the treatment of patients with head and neck cancer. Based on our results that the effect of 5-aza-dCyd is schedule dependent, new drugs should be tested with at least two schedules.

REFERENCES

1. Hong WK, Bromer R. Chemotherapy in head and neck cancer. N Engl J Med 1983; 308: 75-79.
2. Steel GG, Courtenay VD, Peckham MJ. The response to chemotherapy of a variety of human tumour xenografts. Br J Cancer 1983; 47: 1-13.
3. Braakhuis BJM, Snow GB. Activity of conventional drugs in head and neck cancer xenografts. Chapter 6, this thesis.
4. Momparler RL, Bouchard J, Onetto N, Rivard GE. 5-Aza-2'-deoxycytidine therapy in patients with acute leukemia inhibits DNA methylation. Leukemia Res 1984; 8: 181-185.
5. Rivard GE, Momparler RL, Demers J and 4 others. Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. Leukemia Res 1981; 5: 453-462.
6. Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'deoxycytidine. J Biol Chem 1982; 257: 2041-2048.
7. Covey JM, D'Incalci M, Tilchen EJ, Zaharko DS, Kohn KW. Differnce in DNA damage by incorperation of 5-aza-2'-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells. Cancer Res 1986; 46: 5511-5517.
8. Curt A, Allegra CJ, Fine RL, Chao Yeh G, Jolivet J, Chabner BA. Antimetabolites. In: Pinedo HM, Chabner BA, eds. Cancer chemotherapy,

Annual 6. Amsterdam: Elsevier, 1984.
9. Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. Cancer Res 1986; 46, 20-28.
10. Diddens H, Niethammer D, Jackson RC. Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 1983; 43: 5286-5292.
11. Boven E, van der Vijgh WJF, Nauta MM, Schlüper HMM, Pinedo HM. Comparative activity and distribution studies of five platinum analogues in nude mice bearing human ovarian xenografts. Cancer Res 1985; 45: 86-90.

CHAPTER 8

GENERAL DISCUSSION

Results presented in this thesis show that it is possible to grow human head and neck tumours in the athymic nude mouse. The 26% take rate we observe in the first passage is somewhat less than the 29-36% that other authors have found (1-4). Surprisingly, Elprana et al. (5) were able to establish growth in 10 out of 13 tumours attempted. Differences in take rate might be explained by variations in the strain, the health and number of mice, the method of implantation or the evaluation of tumour growth. In this respect it is of importance that a take will have the best chance if the mice are housed in a strictly sterile environment. It is known that when nude mice are exposed to murine hepatitis virus, human tumours fail to grow (6). It is very likely that the tumour take will increase when the mice are placed in the new 'SPF-lab' that is to be built at the Free University campus. In our experience the take rate varied between passages and it sometimes happened that a tumour line suddenly failed to grow.

After a latency period of about 3 to 6 weeks the xenografted tumours doubled their volume in a period of 4 to 15 days. This is in the same order of magnitude as has been described by Galante et al. for local recurrent head and neck tumours in patients (7); these authors measured a median volumes doubling time of 9.5 days.

The question as to whether the characteristics of the tumour are maintained in the mouse is difficult to answer. One can imagine that the tumour undergoes changes associated with progression in the mouse, but this might reflect to some extent the situation in the patient. In some cases a-neuploid subpopulations are selected in the nude mouse (8), which could explain the acceleration of tumour growth in later passages we and others (8, 9) have noted. Whether indeed such selection takes place and which implications this may have for the use of the model for therapeutic studies has yet to be established.

The results of histopathological investigations as reported in this

thesis show that there was no major change in the histopathological characteristics of the tumours after transplantation. Tumour cells still have the possibility to gain characteristics associated with differentiation. Probably due to a lack of vascularization necrosis increased with tumour size. Histopathological control of the transplanted tumours in each passage has been found to be of extreme importance. We have observed that a tumour line was replaced by a lymphoma of murine origin as was proven by LDH-analysis. The cause of the development of this mouse tumour is a matter of speculation, maybe transfection with DNA plays a role, but it is clear that treatment experiments with this tumour would have given false results.

Our studies show that experimental chemotherapy can be studied using human head and neck tumour xenografts in nude mice. As discussed in chapter 7, we can use a panel of tumour lines varying in histology and site of origin. Because of the aforementioned low take rate and the long latency time this model is not suited for chemosensitivity testing of tumours in individual patients. However, the nude mouse model has a significant potential for the screening of new anticancer drugs, as will be discussed below. Our rationale has been to treat mice with tumours with a volume between 50 and 150 mm^3. In our opinion this reflects in a quite reasonable way the situation in patients, where tumours usually have a palpable volume before treatment is started. Therapy results are evaluated by the measurement of changes of tumour volume using vernier calipers. The major advantages of this method are that it is relatively simple, that it is analogous to the way patients tumours are measured, and that the effect on tumour volume can be followed in the course of time. A disadvantage of the method is that it is very time consuming and it does not take into account the proportion of necrosis of rather large tumours. It must be added, however, that at the time of treatment the proportion of necrosis in the tumours is minimal.

Before embarking on chemotherapy studies a number of methodological aspects have to be taken into account. Variables in drug testing are the level of drug dose, the schedule, the mode of administration and how to evaluate the response. In addition, if comparison with clinical results is envisaged the cut-off points of activity are very important (10). Surprisingly, it is usually found that the response rate in the clinic is reproduced by the nude mouse model (11-13). However, in a few cases the

patient and xenograft response do not agree. Our study shows that MTX is unexpectedly inactive in the model as compared to clinical performance; similar findings were observed with 5-FU (11). On the other hand Inaba et al. report over-estimation of the effect of mitomycin C in human gastric tumour lines (14).

A strong feature of the nude mouse model is that there is a variation in response to anticancer drugs, as shown in our studies and those of others (15, 16). This phenomenon is also known from the experience of the clinician. This leads to the conclusion that new drugs must be tested in a panel of tumour lines.

For the evaluation of new drugs some considerations based on the results of the studies presented in this thesis should be taken into account.

1. It is advised to use a panel of tumour lines that reflect a particular patient population. This includes variation of histological subtypes.
2. It is preferable to use lines that are characterized regarding their chemosensitivity pattern to conventional drugs i.e. the drugs usually active for the tumour type concerned. If possible lines should be chosen that reflect the clinical sensitivity pattern.
3. The number of lines that are to be tested will depend on the degree of variability in sensitivity to conventional drugs. The more heterogeneous the pattern of sensitivity the more lines should be used.
4. It is advised to use a maximally tolerated dose which gives weight loss of 5-15% in the first weeks of treatment. So, first toxicity studies are to be carried out. For this purpose nude mice are to be used since the toxicity may be different in normal mice (17).
5. Our results with 5-aza-dCyd show that it is preferable to use at least two schedules: one with a pulse dose every four or seven days and one with multiple injections (1 to 3 per day for at least 5 days).

Using head and neck tumour xenografts we were able to test the activity of some new drugs and preliminary results indicate that these drugs could be of value in patients. However clinical trials with these drugs are needed to analyse the value of the xenograft model in the selection of new drugs. In this respect Boven (16) found that the results obtained with platinum analogs in ovarian xenografts correlated with clinical experiences. Because of the high costs involved, nude mice will never play an important role in primary screening. However, the model is particularly

feasible for secondary screening. Drugs are to be tested on a panel of tumour lines of a given tumour type, analogous to a disease-oriented phase II clinical trial. When the question arises which drug is to be selected for head neck cancer clinical trials, the model might show its value.

As mentioned before a drawback of using the nude mouse model for drug testing is the high cost involved. Also it is labor-intensive and furthermore it takes a few months before an experiment can be evaluated. Therefore we used the xenografts as a tumour source for in vitro testing in a socalled clonogenic assay. In this assay 6 to 10 drugs can be evaluated in about 3 weeks. If the clonogenic assay would predict the in vivo results correctly, the combination of the methods would be a powerful and rapid screening approach. Our results show that in 17 out of 20 xenografts sufficient colony growth was seen to evaluate in vitro drug sensitivity (18). Tumours were considered to be sensitive when the drug concentration required to inhibit colony formation by 50%, was less than 1/10 of the peak plasma concentration in patients. In 32 cases the in vitro data could be compared diredtly with the in vivo results in the nude mouse. When as the cut-off point of activity in vivo of a growth delay of more than two was chosen, the clonogenic assay predicted sensitivity in 4/6 (67%) and resistance in 21/26 (81%) of the cases. Due to this lack of correlation found for drugs like methotrexate, 5-flurouracil and cyclophophamide, this combination of models has some limitations in the testing of new drugs.

Xenografts form an abundant source of tumour tissue outside the human body. Besides their importance for the evaluation of the activity of new drugs, xenografts can be used for the testing of other therapeutic strategies as well for more fundamental research. New developments in therapy like the use of photodynamic effects (3) and monoclonal antibodies directed against tumour cell associated antigens, can be tested in the model. Also, exciting new areas in basic tumour biology, involving oncogenes and growth factors can be explored using the xenograft system (19).

A new approach to cancer chemotherapy is the use of the so-called differentiation inducing agents (20). The concept is that the "cytotoxic" effect of these drugs is actually exerted through the stimulation of tumour cells to differentiate to a more benign cell type, leading eventually to cell death. It is expected that through their mechanism of action these

drugs will cause less side effects. Recently we have started a project investigating the value of differentiation inducers in the treatment of head and neck squamous cell carcinoma. The nude mouse model will be used not only to test the inhibitory effect of the drugs on tumour growth but also will be of help in the development and analysis of differentiation characteristics.

REFERENCES

1. Wennerberg J, Tropé C, Björklund A. Heterotransplantation of human head and neck tumours into nude mice. Acta Otolaryngol (Stockh) 1983; 95: 183-190.
2. Lindenberger J, Ganzer U, Fortmeyer HP. Die Heterotransplantation bösartiger Kopf- und Halsgeschwülste auf thymusaplastische nackte Mäuse. Arch Otorhinolaryngol 1978; 220: 117-128.
3. Hill JH, Plant RL, Harris DM and 4 others. The nude mouse xenograft system: a model for photodetection and photodynamic therapy in head and neck squamous cell carcinoma. Am J Otolaryngol 1986; 7: 17-27.
4. Fantozzi R, Bone R, Fernsten P, Walker L. Upper aerodigestive neoplasm perpetuated in the nude mouse. Laryngoscope 1986; 96: 621-624.
5. Elprana D, Kuijpers W, van den Broek P, Wagener DJT. Growth characteristics of head and neck squamous cell carcinoma in nude mice. Eur J Cancer Clin Oncol 1986; 22: 1211-1222.
6. Kyriazis AP, DiPersio L, Michael JG, Pesce AJ. Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic nude mouse. Int J Cancer 1979; 23: 402-409.
7. Galante E, Gallus G, Chiesa F, Bono A, Bettoni I, Molinari R. Growth rate of head and neck tumors. Eur J Cancer Clin Oncol 1982; 18: 707-712.
8. Wennerberg J. Changes in growth pattern of human squamous-cell carcinomas of the head and neck during serial passages in nude mice. Int J Cancer 1984; 33: 245-250.
9. Mattern J, Haag D, Wayss K. Volm M. Growth kinetics of human lung tumours in nude mice. Expl Cell Biol 1981; 49: 34-40.
10. Martin DS, Stolfi RL, Sawyer RC. Commentary on "Clinical predictivity of transplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy". Cancer Treat Rep 1984; 68: 1317-1318.
11. Fiebig HH, Widmer KH, Fiedler L, Wittekind C, Löhr GW. Development and characterization of 51 human tumor models for large bowel, stomach and esophageal cancers. Dig Surg 1984; 1: 225-235.
12. Osieka R. Studies on drug resistance in a human melanoma xenograft system. Cancer Treat Rev 1984; 11(A); 85-97.
13. Shorthouse AJ, Smyth JF, Steel GG, Ellison M, Mills J, Peckham MJ. The human tumour xenograft-a valid model in experimental chemotherapy? Br J Surg 1980; 67: 715-722.
14. Inaba M, Tashiro T, Kobayashi T and 6 others. Evaluation of response rates to various antitumor agents of human gastric tumors implanted in nude mice. Gann 1986; 77: 190-196.

15. Nowak K, Peckham MJ, Steel GG. Variation in response of xenografts of colo-rectal carcinoma to chemotherapy. Br J Cancer 1978; 37: 576-584.
16. Boven E. Human ovarian cancer xenografts in nude mice. Thesis Free University, Amsterdam, the Netherlands. Amsterdam: Rodopi, 1986.
17. Houchens DP, Johnson RK, Gaston MR, Goldin A. Toxicity of cancer chemotherapeutic agents in athymic (nude) mice. Cancer Treat Rep 1977; 61: 103-104.
18. Boerrigter GH, Heinerman ECM, Braakhuis BJM, Snow GB. Chemosensitivity of human head and neck cancer xenografts in the clonogenic assay and in nude mice. Br J Cancer 1986; 54: 53-59.
19. Totosyan AG, Galetzki SA, Kisseljova NP and 6 others. Oncogene expression in human tumors. Int J Cancer 1985; 35, 731-736.
20. Santorelli AC. Malignant cell differentiation as a potential therapeutic approach. Br J Cancer 1985; 52: 293-302.

SUMMARY

There is a need for new anticancer agents in the treatment of patients with a tumour in the head and neck region. This study was initiated in order to investigate the potential value of an experimental model which can be used in the selection of agents for head and neck cancer phase II clinical trials. Because of the promising results with tumour xenografts transplanted in athymic nude mice, we decided to use this model for this study. In this thesis various aspects of human head and neck cancer xenografts growing in nude mice are discussed.

As presented in chapter 2, we found that 26% of 129 human head and neck tumours attempted, showed growth in the first passage. Serial transplantation was possible in about half of tumours that initially demonstrated growth. Poorly differentiated squamous cell carcinoma tended to grow more readily than more differentiated tumours and the same applies for lymph node metastases as opposed to primary tumours. In a majority of the tumour lines (4/6) the tumour volume doubling time decreased when the number of passages increased. Microscopical examination of transplanted tumours revealed that xenografting caused no major changes in the histology. No signs of metastasis was observed in any of the inspected mice.

Because of the low take rate of the head and neck tumours it was investigated, as described in chapter 3, whether immunosuppression of the nude mouse could lead to an enhancement of the tumour take rate. Immunosuppression was performed by intraperitonal injection of a well tolerated dose of cyclophosphamide 24 hrs before implantation of tumour fragments. Using this dose of cyclophosphamide we could demonstrate an inhibition of the function of natural killer cells, a cell type presumed to be involved in tumour growth control. Immunosuppression with cyclophosphamide did not lead to an increase of the take of head and neck tumours These results are in contrast with those obtained with ovarian tumour lines; in some of these lines a significant increase of the take rate was observed.

In chapter 4 the activity of maximum tolerated doses of three drugs that are clinically effective in head and neck cancer is described. Cisplatin and bleomycin appeared to be effective agents in the model as

well, while methotrexate showed essentially no activity. For two tumours the reaction of the xenografts to methotrexate did not correlate with the chemotherapy results in the patients from whom the xenografts were derived. The patient tumours responded to treatment with this drug while the xenografts showed no sensitivity at all.

In chapter 5 it is shown that xenografts can be used for the study of resistance mechanisms. In this case methotrexate resistance was analysed and the results show that a lack of effect is probably not due to i) a plasma pharmacokinetic factor in the nude mouse and ii) overproduction of dihydrofolate reductase, the target enzyme of methotrexate.

In chapter 6 five drugs were analysed with clinical activity. Bleomycin was the most active drug (response in 4/9 lines), and 5-fluorouracil and cyclophosphamide were less active (moderate responses in respectively 4/7 and 4/6 lines). Methotrexate and cisplatin were minimally or not active. The inactivity of cisplatin as opposed to the results reported in chapter 4 is likely to be a dose related effect, since we were forced to use a lower dose in the latter experiments.

In chapter 7 the activity of some new drugs is reported. Platinum analogs such as carboplatin, spiroplatin and JM-40 were tested in platinum insensitive lines and no superior effect of the analogs as compared to cisplatin was observed. When tested in a single line, 5-aza-2'-deoxycytidine as well as 5'-deoxy-5-fluorouridine showed a better activity than conventional drugs, making these drugs of potential value for patients with head and neck cancer.

In chapter 8 conclusions are drawn about the potential of the model. Because of the low take rate and the long latency time the model is not suited for chemosensitivity testing in individual patients. Also, because of the high costs involved the nude mouse will never play an important role in the primary screening of new drugs. However the model is very likely to be of relevance in the secondary screening of new drugs. Testing has to be done in a panel of head and neck tumour lines, analogous to a disease oriented phase II clinical trial. When the question arises which drug is to be selected for trials in head and neck cancer patients, the model might show its value. Further studies must be awaited to draw definitive conclusions about the relevance of the model.

SAMENVATTING

Er is behoefte aan nieuwe cytostatica voor de behandeling van patiënten met een hoofd- halstumor. Naast een zo goed mogelijk antitumor effect is het gewenst dat nieuwe stoffen vooral minder bijwerkingen hebben dan de op dit moment gebruikte. Om erachter te komen of een nieuw cytostaticum actief is bij patiënten met een hoofd- halstumor, vindt altijd een klinische (zogenaamde fase II) studie plaats. Bij een dergelijke studie is het mogelijk dat groepen patiënten met een inactief middel behandeld worden. Zij worden dan onnodig blootgesteld aan toxische bijverschijnselen.

Dit onderzoek is gestart met de vraag of het mogelijk is een preklinisch model te ontwikkelen, waarvan de resultaten gebruikt zouden kunnen worden bij het selecteren van cytostatica voor een fase II studie bij patiënten met een hoofd- halstumor. Zo'n model zou dan moeten voorkomen, dat patiënten onnodig blootgesteld worden aan inactieve middelen. Wij besloten een in vivo model te kiezen waarbij de naakte thymusloze muis als gastheer fungeert voor humane hoofd- halstumoren. Door het ontbreken van de thymus heeft de naakte muis zeer weinig lymfocyten met T-cel kenmerken en deze immuundeficiëntie heeft tot gevolg dat de muis weefsel van andere species (xenotransplantaten), zoals ook menselijke tumoren accepteert. Deze immuundeficiëntie maakt het dier echter ook extreem gevoelig voor ziektekiemen; de muis moet dan ook in een omgeving zonder kans op infectie met pathogene microörganismen gehouden worden. Dit impliceert dat bakken, voer, water en alles wat verder met de muis in contact komt steriel moeten zijn. Het is duidelijk dat het houden van een naakte muizenkolonie een kostbare en tijdrovende zaak is.

In hoofdstuk 2 wordt beschreven hoe het implanteren van tumoren plaats vond. Na ontvangst van een tumor van een patiënt, werd deze in stukjes gesneden en aan elke zijde van de muis subcutaan in de flank geïmplanteerd. In 26% van de 129 gevallen waarbij een tumor geïmplanteerd werd, sloeg de tumor aan: groei werd waargenomen. In ongeveer de helft van de gevallen waar groei gezien werd in de eerste (mens naar muis) passage, was het mogelijk de in de muis groeiende tumor steeds van de ene groep muizen over te zetten in een andere. Wanneer meer dan 4 van dergelijke passages

mogelijk waren met een bepaalde tumor, dan werd er gesproken van een tumorlijn. Er werden sterke aanwijzingen gevonden, dat slecht gedifferentieerde plaveiselcelcarcinomen vaker in de eerste passage aanslaan dan de beter gedifferentieerde carcinomen. Ook lymfekliermetastasen leken vaker te groeien dan de primaire tumoren. In vier van de zes tumorlijnen nam de gemiddelde verdubbelingstijd van de tumoren af naarmate de tumor meer passages doorlopen had. Na vier tot zes passages stabiliseerde de groeisnelheid zich. Microscopisch onderzoek toonde aan dat de transplantaten qua histologie niet wezenlijk verschillen van de oorspronkelijke tumor; de differentiatiegraad van plaveiselcelcarcinomen veranderde niet. Metastasering, een kenmerk van maligniteit, kon in tumordragende muizen niet aangetoond worden.

Het feit, dat slechts een minderheid van de geïmplanteerde tumoren gaat groeien in de naakte muis was aanleiding voor een experiment, dat tot doel had d.m.v. extra onderdrukking van het immuunsysteem van de muis de groei van hoofd- hals tumoren te bevorderen. Wij konden aantonen dat cyclophosphamide 24 uur nadat het intraperitoneaal is toegediend een remmend effect heeft op de activiteit van natural killer cellen. Er zijn sterke aanwijzingen, dat dit type cellen betrokken is bij de afstoting van tumorweefsel. Cyclophosphamide 24 uur voor de transplantatie gegeven, veroorzaakte geen toename van het percentage hoofd- halstumoren dat aansloeg in de naakte muis. Bij ovarium tumoren werd verassenderwijs wel een positief effect op het aanslaan van de tumoren waargenomen.

In hoofdstuk 4 worden de resultaten besproken van de behandeling van tumordragende muizen met de drie cytostatica, die bij patiënten met hoofd-halstumoren het meest werkzaam zijn. Cisplatine en bleomycine bleken actieve stoffen te zijn in de meerderheid van de geteste tumorlijnen, terwijl methotrexaat geen of nauwelijks activiteit vertoonde in een 11-tal lijnen. Er konden tumorlijnen gemaakt worden van tumoren van 2 patiënten, die na de transplantatie behandeld werden met methotrexaat. De gevoeligheid van de tumor in de patiënt voor methotrexaat correleerde niet met de minimale activiteit van deze stof op de getransplanteerde tumoren.

Naast de studies naar het effect van cytostatica kan het model ook gebruikt worden voor het bestuderen van resistentie-mechanismen. In hoofdstuk 5 wordt aangetoond dat de ongevoeligheid voor methotrexaat van de getransplanteerde tumoren waarschijnlijk niet veroorzaakt wordt door i) een

verschil in farmacokinetiek van methotrexaat tussen mens en muis en ii) een overproductie van dihydrofolaat reductase, het doelwit enzym van methotrexate.

In hoofdstuk 6 worden de resultaten beschreven van vijf cytostatica waarvan bekend is dat ze in 20-40% van de patiënten antitumor activiteit vertonen. Bleomycine was het meest actief, met een goede werkzaamheid in vier van de negen lijnen, terwijl 5-fluorouracil en cyclophphosphamide matig actief waren in respectievelijk vier van de zeven en vier van de zes lijnen. Methotrexaat en cisplatine waren nauwelijks of niet actief in een 10-tal lijnen. Het ontbreken van activiteit van cisplatine komt niet overeen met de resultaten zoals beschreven in hoofdstuk 4. Het is aannemelijk, dat dit verschil veroorzaakt wordt door het feit dat wij bij de in hoofdstuk 6 beschreven experimenten gedwongen waren de totale dosis cisplatine te verlagen.

In hoofdstuk 7 worden de resultaten met nieuwe cytostatica beschreven. De activiteit van platinum-analoga, zoals carboplatin, spiroplatin en JM-40 werd onderzocht in tumorlijnen die ongevoelig waren voor cisplatine. Geen van deze nieuwe platinum-analoga vertoonde een beter antitumor effect dan cisplatine. 5-aza-2'-deoxycytidine en 5'-deoxy-5-fluorouridine bleken ieder in een tumorlijn actiever te zijn dan alle conventionele cytostatica. Deze resultaten geven aan dat deze twee stoffen mogelijk van waarde zijn bij patiënten met een hoofd-halstumor.

De waarde van het model wordt in hoofdstuk 8 besproken. Omdat het percentage tumoren, dat uiteindelijk uitgroeit tot een tumorlijn vrij klein is en het vrij lang duurt voordat een chemotherapie-experiment geëvalueerd kan worden, is het model niet of nauwelijks van waarde voor de behandeling van de individuele patiënt. Ook zal het model vanwege de hoge kosten nooit een rol gaan spelen bij het zogenaamde "primary" screenen d.w.z. het testen op antitumor activiteit van stoffen waarvan niets bekend is. Het model zal waarschijnlijk waardevol zijn voor het "secondary" screenen van cytostatica. Stoffen, welke door andere, goedkopere modellen als interessant bestempeld zijn of welke actief zijn bij andere tumortypen worden dan getest in een panel van hoofd- halstumorlijnen. De aldus verkregen resultaten kunnen betrokken worden bij de beslissing welke cytostatica getest moeten gaan worden in fase II studies bij patiënten met hoofd-halstumoren. Op dit moment zijn meer resultaten nodig om de definitieve

waarde van het naakte muizemodel voor het "secondary" screenen van nieuwe cytostatica te bepalen.

PUBLICATIONS

The following publications resulted from the studies described in this thesis.

1. Braakhuis BJM and Snow GB. Chemotherapy of human head and neck tumours transplanted in athymic nude mice (abstract). Expl. Cell Biol. 50: 348, 1982.
2. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Sneeuwloper G and Snow GB. Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cis-platinum, bleomycin and methotrexate. Br. J. Cancer 48: 711-716, 1983.
3. Braakhuis BJM, Sneeuwloper G and Snow GB. Chemotherapy of human head and neck cancer xenografts in athymic nude mice (abstract). Z. Versuchstierk. 25: 203, 1983.
4. Braakhuis BJM, Sneeuwloper G and Snow GB. The establishment of human head and neck tumour xenografts (abstract). Z. Versuchstierk. 25: 203, 1983.
5. Braakhuis BJM, Heinerman ECM, Schoevers EJ and Snow GB. Minimal activity of methotrexate against human head and neck cancer xenografts in nude mice (abstract). 2nd European conference on clinical oncology and cancer nursing, Amsterdam, 1983.
6. Braakhuis BJM, Sneeuwloper G and Snow GB. Chemotherapy of human head and neck tumours transplanted in athymic nude mice. In: Immune-deficient animals. Ed. B.Sordat, p. 301-303, 1984. Karger, Basel.
7. Braakhuis BJM, Sneeuwloper G and Snow GB. The potential of the nude mice xenograft model for the study of head and neck cancer. Arch. Otorhinolaryngol. 239: 69-79, 1984.
8. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Boerrigter GH and Snow GB. Unexpected minimal activity of methotrexate in human head and neck cancer xenografts (abstract). International conference on head and neck cancer, Baltimore, USA, 1984.
9. Braakhuis BJM and Snow GB. Nude mice model as a predictive assay in head and neck cancer. In: Head and Neck Cancer. Vol. 1. Eds: Chretien, P.B. et al. pp. 421-424, 1985. Decker, Philadelphia.

10. Braakhuis BJM, Leyva A, Schoevers EJ, Boerrigter GH, Schornagel JH and Snow GB. Lack of effect of methotrexate on human head and neck tumours transplanted in athymic, nude mice. Acta Otolaryngol (Stockh.) 99: 208-213, 1985.
11. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Boerrigter GH and Snow GB. Oral cancer in the nude mouse xenograft model (abstract). J. Oral Pathol. 14: 67, 1985.
12. Braakhuis BJM, Leyva A, Pinedo HM and Snow GB (abstract) Therapetic effect of 5-aza-2'-deoxycytidine in human head and neck tumour xenografts. 5th international conference on immune-deficient animals, Copenhagen, 1985.
13. Braakhuis BJM, Nauta MM, Romijn JC, Rutgers DH and Smink T. Enhanced success rate of transplantation with human tumors in cyclophosphamide treated nude mice. JNCI 76: 241-245, 1986.
14. Braakhuis BJM, Leyva A, Pinedo HM and Snow GB. Therapeutic effect of 5-aza-2'-deoxycytidine in human head and neck tumour xenografts. In: Immune deficient animals. Ed. J. Rygaard, 1986, Karger, Basel. In press.
15. Braakhuis BJM, Leyva A, Pinedo HM and Snow GB. Antitumor effect of 5-aza-2'-deoxycytidine (5-aza-dCyd) in human head and neck cancer xenografts (abstract). Proc. Am. Ass. Cancer Res. 299, 1986.
16. Braakhuis BJM, Heinerman ECM, Boerrigter GH and Snow GB. Chemosensitivity of human head and neck (H&N) cancer xenografts in the clonogenic assay and in nude mice (abstract). 5th NCI-EORTC symposium on new drugs in cancer therapy, Amsterdam, 1986.
17. Braakhuis BJM, Leyva A and Snow GB. Activity of three new antimetabolites in human head and neck cancer xenografts (abstract). 5th NCI-EORTC symposium on new drugs in cancer therapy, Amsterdam, 1986.
18. Braakhuis BJM, Leyva A, Pinedo HM and Snow GB. Activity of six new drugs in head and neck cancer xenografts. ESO-monographs on human tumor xenografts in anticancer drug development. In press.
19. Braakhuis BJM and Snow GB. Activity of conventional drugs in head and neck cancer xenografts. ESO-monographs on human tumour xenografts in anticancer drug development. In press.
20. Heinerman ECM, Braakhuis BJM and Snow GB. Human head and neck cancer xenografts as a tumour source in the human tumour stem cell assay (abstract). 2nd European conference on clinical oncology and cancer nursing, Amsterdam, 1983.
21. Heinerman ECM, Braakhuis BJM, Boerrigter GH and Snow GB. Successful in vitro growth of human head and neck cancer after transplantation in athymic nude mice. Arch. Otorhinolaryngol. 241: 225-232, 1985.
22. Boerrigter GH, Heinerman ECM, Braakhuis BJM and Snow GB. Human oral cancer in the clonogenic assay (abstract). J. Oral Pathol. 14: 66, 1985.
23. Heinerman ECM, Boerrigter GH, Braakhuis BJM and Snow GB. Drug sensitivity testing of human head and cancer in the clonogenic assay (abstract). Clinical otolaryngology 10: 228, 1985.
24. Boerrigter GH, Heinerman ECM, Braakhuis BJM and Snow GB. Chemosensitivity of human head and neck cancer xenografts in the clonogenic assay and in nude mice. Br. J. Cancer 54: 53-59, 1986.
25. Peters GJ, Braakhuis BJM, Snow GB and Pinedo HM. Improved therapeutic effect of 5'deoxy-5-fluorouridine (5'dFUR) in 5-fluorouracil (5FU) insensitive experimental human and murine tumors (abstract). Proc. Am. Ass. Cancer Res. 300, 1986.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 7 april 1955 te Hoorn. Na de gymnasium ß opleiding op het Pius X college te Beverwijk (eindexamen in 1973) begon zijn studie biologie aan de Vrije Universiteit te Amsterdam. In 1977 werd het kandidaatsexamen medische biologie gehaald. Het doctoraal-examen werd in april 1981 afgelegd, met immunologie als hoofdvak en histologie en farmacologie als bijvakken. Sinds april 1981 is hij werkzaam bij de vakgroep Keel-, neus- en oorheelkunde en Audiologie van de Vrije Universiteit te Amsterdam. Van 1981 tot 1986 werkte hij aan een door het Koningin Wilhelmina Fonds gesubsidieerd project.

STELLINGEN

1. Bij de beslissing, welke cytostica gekozen moeten gaan worden voor een fase II studie met patiënten met een hoofd- halstumor kan het model, dat gebruik maakt van naakte muizen, waarbij dit type tumor groeit, een belangrijke rol gaan spelen.

2. Het onderdrukken van het immuunsysteem van naakte muizen middels een injectie met cyclophosphamide, veroorzaakt geen toename van het percentage humane hoofd- halstumoren, dat aanslaat bij deze dieren.

3. Methotrexaat, gegeven als dagelijkse of wekelijkse intraperitoneale injecties, heeft nauwelijks of geen groeivertragend effect op humane hoofd- halstumoren, die als xenotransplantaten groeien in naakte muizen.

4. Vanwege het lage percentage tumoren dat groeit en de lange tijd die nodig is om gegevens over de gevoeligheid voor cytostatica te verkrijgen, is het naakte muizemodel van zeer beperkte waarde voor de individuele patient met een hoofd- halstumor.

5. Bij analyse van de keratine subtypen van het mondholte-epitheel, dient men er zich van bewust te zijn, dat de relatieve aanwezigheid van deze subtypen niet alleen per lokalisatie maar ook per individu variëert (Clausen et al. J Invest Dermatol 86, 249, 1986).

6. De afwezigheid van een antitumor effect van methotrexate op humane hoofd- halstumoren, die groeien in de naakte muis wordt niet veroorzaakt door een defect aan het carrier-eiwit, dat zorgt voor actief transport van methotrexaat.

7. Gezien de verhoogde expressie van de epidermale groeifactor receptor op plaveiselcelcarcinomen, is het van groot belang de diagnostische waarde van dit membraaneiwit vast te stellen (Ozanne et al. J Pathol 149, 9, 1986).

8. Follikulair dendritische cellen ontwikkelen zich uit reticulumcellen.

9. Het meten van veranderingen in celmetabolisme met behulp van ^{31}P kernspinresonantie spectroscopie kan de basis worden van een op de individuele patiënt gerichte antikanker therapie.

10. Gezien de belangrijke nieuwe ontwikkelingen in de biologische wetenschappen verdient het aanbeveling de eisen, die aan het eindexamen biologie voor middelbare scholieren gesteld worden en die sinds het invoeren van de mammoetwet hetzelfde zijn gebleven, aan te passen.

11. Het is onbegrijpelijk, dat als neveneffect van het invoeren van de 2-fasenstructuur, academici, die het oude curriculum doorlopen hebben, bij het in dienst treden van een universiteit, genoegen moeten nemen met een salaris, dat weinig meer is dan het wettelijk minimumloon.

12. Er moet gevreesd worden, dat de toename van het aantal muizestammen (op dit moment 900) een factor is, die het gebruik van proefdieren zal stimuleren (Current Contents 29, 50, p14, 1986).

13. Voor de moderne lichtgewichtkampeerder is 'terug naar de natuur' slechts een relatief begrip; bij deze hobby is het gebruik van kunstvezels schering en inslag.