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A STUDY OF THE FACTORS WHICH AFFECT THE GROWTH OF TUMOUR CELLS AT DISTANT SITES

ANDREW S. JACK.

A thesis submitted in fulfilment of the regulations for the degree of Ph.D. in the Faculty of Medicine.

Department of Pathology,
University of Glasgow,
Glasgow Royal Infirmary,
GLASGOW. G4 0SF

June 1986

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to express my thanks to Professor R.B. Goudie for his interest and patient supervision of this work. I would also like to thank Dr. H. Simpson and my colleagues in the Department of Pathology for their encouragement.

I am most grateful to Ms. Karen L. McVeigh for her skilled technical assistance and to the staff of the Pathology Department Animal Unit for their assistance in animal breeding.

Professor A.S.G. Curtis provided invaluable assistance in teaching me the rudiments of cell culture, Dr. C. Evans gave me much helpful advice in the handling of B16 cells. Miss E. MacKenzie and Dr. G. Birnie (Beatson Institute) gave freely of their time and experience in operating the fluorescence activated cell sorter and advising in the interpretation of the results.

Mr. T. Parker (Medical Illustration Department) processed the black and white photographs and the manuscript was typed by Mrs. I.O. Main. Finally, I would like to express my gratitude to my brother Shanks Jack for his efforts in preparing the computer drawn graphs.

This work was financed by grants from the Greater Glasgow Health Board and by University General Funds. For most of the period of this study I was supported by a Wellcome Trust Lectureship.

DECLARATION

The part played by many of my colleagues and others in this study has been acknowledged above. In particular the assistance of Ms Karen McVeigh allowed experiments to proceed while I was engaged in other departmental duties. However, all the experiments described in this thesis were initiated by myself and I personally performed or supervised all the technical work involved.

LIST OF FIGURES AND APPENDICES

APPENDICES

ABBREVIATIONS

EDTA	- Ethylene diamine tetra-acetic acid.
FITC	- Fluoroscein Isothiocyanate
Hepes	- N-2 - hydroxyethyl piperazine N-3 ethane sulphonic acid.
H-2	- Mouse major histocompatability locus.
HLA-DR	- Human class II major histocompatability gene.
IV	- Intravenous (injection).
IP	- Intraperitoneal (injection).
M	- Molar.
MBq	- Megabecqurel i.e. disintegrations x 10^6 s^{-1}.
MCA45	- Monoclonal antibody MRC-OX3.
MCM64	- Monoclonal antibody 5041 - 16.3.
MEM	- Minimum Essential Medium.
PBS	- Phosphate buffered saline.
Tris	- Tris (hydroxymethyl) aminomethane.
KD	- Kilodaltons.
SE	- Standard error. Numerical results are expressed as $x \pm y$ where x is the mean and y the standard error of the mean.

GENERAL SUMMARY

The aim of this thesis is to investigate some of the factors which affect the growth of metastasising cells at distant sites. Previous experiments, mostly involving intravenous injection of cultured murine B16 melanoma and other tumour cell lines, have suggested that sub-populations of cells exist within tumours which are capable of metastasis, sometimes to specific organs. One criticism of these studies is that growth at a distant site is but one characteristic required for a cell to successfully form a metastatic deposit. The cell must also express other phenotypic characteristics such as motility and invasion and in some cases evasion of host defences.

Much of the experimental work in this thesis makes use of the B16 melanoma F10 and F1 cell lines. The F10 cell line was derived many years ago by repeated *in vivo* passage through the lungs of syngeneic mice while the F1 cell line was passaged *in vivo* only once and has been maintained exclusively *in vitro*. In this study it was found that the F10 cell line formed tumours at a high rate, exclusively in the lungs, whereas the F1 cell line was much less metastatic and selective in its site of growth. The use of radio-labelled cells showed that the F10 cells were more avidly trapped in the lungs than the F1 cells but the difference in lung trapping between the two cell lines was less marked than the difference in lung tumour formation. These results broadly confirm the results of earlier studies and show that despite prolonged culture the cell lines have been stable with respect to these properties.

In contrast to the results of intravenous injection, when F1 and F10 cells were injected into the peritoneal cavity it was found that there was no difference in the number of tumours produced. Each cell line was then subjected to repeated intraperitoneal passage to see whether passaged cells formed more local tumours when injected peritoneally, whether such cells would home to the peritoneal cavity following intravenous injection, and if passage in the peritoneum would effect lung homing properties particularly of the F10 cell line. After 16 passages an F10 cell line was produced which grew more readily in the peritoneal cavity but did not produce abdominal tumours when injected intravenously: lung tumour formation was unaffected. When F1 cells were passaged it was found that after 8 passages cell growth in vitro and in the peritoneal cavity was so greatly reduced that in only one of three sets of experiments was it possible to proceed beyond the eighth passage. The set of F1 cells which reached passage 16 continued to show poor growth in vitro and after intraperitoneal injection but surprisingly produced a much larger number of lung tumours following intravenous injection than the parent cell line. This latter change was not accompanied by increased cell trapping in the lungs.

By way of comparison a benign virus-induced salivary tumour was studied. This model was limited by failure to culture the tumour cells in vitro. When a cell suspension derived from this tumour was injected intraperitoneally no tumours resulted. Thus the benign nature of this tumour may have been partly due to an inability to grow at a distant site.

An attempt was made to correlate adhesion of B16 and salivary tumour cells to tissue sections with their sites of growth but in contrast to previous studies no such correlation was found.

Mechanisms to account for these results are discussed with particular reference to the effects of selection, trapping by the vasculature and the influence exerted by local tissue environments on tumour cells. While these mechanisms are often difficult to separate experimentally there was some evidence that local environments may play a larger part than hitherto realised in controlling the growth of metastasising tumour cells.

Little is known of the molecular basis of tumour cell metastasis, though attempts to identify cell surface proteins and more recently the genes involved in the process have met with some success. Since the histo-compatability proteins are known to participate in a wide range of cellular interactions, they may be involved in the process of metastatic tumour growth. Accordingly, monoclonal antibodies to the major histocompatability complex (MHC) K and D proteins and the class II I-A protein were used to study expression of these molecules in the B16 F1 and F10 cells. Using a fluorescence activated cell sorter it was found that expression of these antigens was greater in the F10 than in the F1 cell. Treatment of the cells with retinoic acid caused increased expression of both class I and class II molecules, the increase being greater in the F1 cells. However attempts to study the changes in metastatic potential after retinoic acid induction were thwarted because of unexpected effects

on cell division. Retinoic acid produced a marked increase in the lung trapping of F1 cells, at concentration which stimulated MHC expression. However, the opposite was true of F10 cells where a decrease in lung trapping was found after retinoic acid treatment. These results are discussed in the context of the various MHC effects which are described in other tumour models.

The role of histocompatability proteins was further studied by testing for strain related restriction effects such as those demonstrable in the immune system. Intravenous injection of F10 cells into a semi-allogeneic recipient produced a large decrease in the number of lung lesions but not in the number of peritoneal deposits following intraperitoneal injection or in trapping of radio-labelled cells. Using a hybrid which was homozygous at H-2 but not at any other locus it was found that the decrease was not due to the H-2 locus but to other strain-related polymorphisms. On review of the literature it was apparent that this effect has been observed elsewhere but has not been previously commented on.

In view of their great diversity it is possible that the non-H-2 class I genes especially could be important mediators of the cell interactions which govern the growth and positioning of metastasis. Unfortunately further investigation is inhibited by lack of suitable reagents and strains of animals of appropriate genotype.

CHAPTER ONE

GENERAL INTRODUCTION.

CHAPTER 1

GENERAL INTRODUCTION

The work described in this thesis concerns the factors which control the growth of metastasising tumour cells at distant sites. There are two distinctive and complementary approaches to this problem. The first entails the search for sub-populations of cells within malignant tumours which are able to grow at distant sites. This is one aspect of the general question of tumour progression. The second approach concerns the phenotype of metastasising cells. Many cellular characteristics correlate with the ability of tumour cells to form metastasis at distant sites. Some of these are of a general nature, for example, adhesion and motility, while others such as interaction with platelets relate to identifiable steps in the process of metastasis. In this chapter both these approaches to the problem will be considered in order to place the experimental work described in chapters 2 and 3 in a wider context.

1.1. Clinical Studies of Metastasis

The origin of the study of metastasis lies in human pathology and with a number of important observations. It has long been recognised that many malignant tumours metastasise to particular sites (Willis, 1952). It is clear that two factors govern the observed pattern. Metastasis to local lymph nodes or to the liver,in the case of gastrointestinal tumours is explicable on the basis of lymphatic or blood flow (Sugarbaker, 1981, Sugarbaker, 1979). However, the site preference of many tumours is not explained by this mechanism. An example is seen in figure 1 of a malignant melanoma which colonised the duodenum but

did not spread beyond the gastro-duodenal junction. The tendency for malignant lymphomas to colonise symmetrical structures such as the earlobes, conjunctiva or breasts has also been described (Morgan 1971). These observations lead to the hypothesis that site specific factors are present which favour or retard the growth of potentially metastatic cells. Further support for this hypothesis is found in the observed variation in growth rates of tumour cells at different sites. Metastatic breast carcinoma cells may become apparent many years after the removal of a primary tumour (Adair et al, 1974, American Joint Committee for Cancer Staging, 1978, Brinkley and Haybittle, 1975, Pawlias et al, 1958) which suggests the possibility that local factors may inhibit growth of these cells. There is also some evidence that the growth may be accelerated by local trauma and subsequent healing (Per Hagopian et al, 1978).

A second question raised by clinical studies is the nature of the relationship between cells which form metastases and the general population of cells in the primary tumour. In some cases it has been found that the probability of finding metastasis is closely related to the size of a primary tumour (McNeal et al, 1986). This is not an invariable rule; many tumours may be undetectable at the primary site, but, present with multiple metastasis. For example, in tumours of the head and neck, size is predictive of the formation of metastasis by carcinoma of the mouth but not by carcinoma of the pharynx (Lindberg, 1972). This suggests that the tumour phenotype which favours the formation of metastases may differ from that which favours local growth and hence leads to a hypothesis

that metastasising sub-populations of cells may be present in a primary tumour. This idea is supported by many observations of differences in karyotype (Vindelov et al, 1980, Shapiro et al,,1981, Abeloff et al, 1979), receptors (Brennan et al, 1979) and surface antigens (Dexter and Calabresi, 1982) between tumours and their metastases.

1.2. Experimental Studies of Metastasis.

The clinical observations described above form the basis of the experimental study of metastasis. The studies which will be described seek answers to the following questions. Are distinct metastasising cell sub-populations present within a primary tumour ? Are sub-populations present which show a preference for growth at a particular distant site ? What properties must a tumour cell have to allow it to form a metastasis, either in general or at a particular site ?

a) The Evolution of Metastasising Cells from a Primary Tumour.

The multistage model of carcinogenesis has become widely accepted. Three stages are recognised: initiation, promotion and progression (Farber & Cameron, 1980, Medline & Farber, 1981). The initiation and promotion steps can be readily demonstrated in mouse skin where the efficiency of the carcinogen is greatly enhanced by subsequent application of croton oil (Berenblum, 1941). These effects are also seen in mouse liver where partial hepatectomy increases the yield of hepatomas following a single dose of a chemical carcinogen (Solt et al, 1977). In cattle it has been postulated that food constituents may promote the effects of papilloma virus infection in causing gastric carcinoma (Jarrett et al, 1978). It is reasonable to

regard the initiation event as a stage of genetic injury. Many initiating agents may give rise to electrophilic agents capable of reacting with nucleic acids (Yahagi et al, 1975, DeBaun et al, 1970). In one case the genetic change following carcinogenesis has been shown to be a mutation of the ras proto-oncogene (Marshall et al, 1984). Promotion can be seen as a growth stimulus which may act by "fixing" the genetic change caused by the initiator or simply by stimulating cell division. The phorbol esters are important tumour promoters and probably act through the protein kinase C pathway in competition with endogenous diacylglycerol (Moolenaar et al, 1984).

The formation of metastasis is regarded as belonging to the third stage in carcinogenesis. In contrast to intitiation and promotion, progression is much more difficult to define in cellular or molecular terms. It is uncertain whether the genetic changes of the initiation step are sufficient or whether further change must occur, with the evolution of sub-populations before progression to metastasis. Many of the basic concepts of tumour progression were described by Nowell (Nowell, 1976). The model envisaged is one of evolution by natural selection; multiple variants being generated by mutation, most of which are eliminated, while a few persist and replace the progenitor cell population. Fundamental to this view is the idea that tumours have an acquired but inherited degree of genetic instability. There is some evidence for this view in the increase in mitotic errors seen in neoplastic cells (Hellstrom et al, 1963) and the correlation of karyotypic abnormalities with increased malignancy

(Rowley, 1975). Although some of the mechanisms of DNA repair are well understood (Lindahl, 1982) little is known about the overall control of genomic stability and how this mechanism could be impaired in neoplastic cells. An alternative view of tumour progression is that local environmental influences may modulate tumour cell behaviour without the need for further genetic change. There is little direct evidence for epigenetic factors of this type in tumour progression although in some cases the neoplastic behaviour of cells may be controlled by the environment even in the presence of gross karyotypic abnormalities. The regulatory effects of embryonic tissue on teratocarcinoma cells is a good example (Pierce, 1983). In a recent review Hart, (1984) proposed six general principles of tumour progression. These are (1) individual characteristics progress independently (2) tumours evolve at different rates (3) progression is independent of tumour growth (4) progression may be continuous or discontinuous (5) progression follows one of the alternative paths of development (6) progression does not always reach an end point within the lifetime of the host. It is difficult to see what these principles add to the understanding of the problem of tumour progression. Principles 1 - 4 appear to be simple restatements of the idea that progression is a function of genetic change. In principle 5 the author does not explain what he means by alternative pathways of development. The sixth principle merely alludes to the fact that in vitro cell lines may continue to change over very long periods of time.

From the point of view of the study of metastasis the

question of tumour progression is largely encompassed by the question as to whether or not metastases arise from distinctive cell sub-populations within the primary tumour. If such sub-populations are demonstrated then it becomes relevant to ask how they were initially generated.

These questions will be approached in the following way. (1) The B16 mouse melanoma model will be described. Work with this model and its interpretation constitutes a very large proportion of the literature on this subject. (2) Other models of metastasis in mice will be discussed. (3) The arguments for and against the concept of heterogeneity and will be presented. (4) Possible mechanisms of generation of heterogeneity will be described.

b) The B16 mouse melanoma

The B16 melanoma is a spontaneous tumour which arose in 1954 in a C57 BL6 mouse. It was initially passaged *in vivo* before it was permanently established in culture. The widespread use of this model in studying the process of metastasis stems from a much quoted experiment (Fidler & Kripke, 1977, Poste & Fidler, 1980). In this experiment 17 individual clones were isolated from a sample of B16 cells. Cells from each clone were injected intravenously into groups of syngeneic mice and the number of tumour deposits compared with those produced by the uncloned parent cell line. This experiment showed that most of the clones differed in their ability to produce tumours as compared to the parent cells. Sub-clones derived from some of the original clones were identical in tumour forming ability. From these experiments it was concluded that the

parent cell line contained a heterogeneous population of cells differing in metastatic potential which was stably inherited.

Injection of cells from the parent cell line produced pulmonary lesions in every animal tested. Lesions were also apparent in the ovary, liver, gut, lymph nodes, adrenal glands, heart, kidney and nasal sinuses (Fidler and Kripke, 1977). Earlier work with B16 cells had produced cell lines selectively metastatic to particular organs. A lung selective line was produced (Fidler, 1973) by the in vivo culture of cells derived from pulmonary lesions followed by intravenous injection into the syngeneic host. Ten cycles of this type were required to produce a highly selective line designated B16 F10. Cells derived from the first passage B16 F1 produced many fewer lesions when injected intravenously and are less lung selective. Using an identical method, cell lines with selectivity for liver (Tao et al, 1979) ovary (Brunson & Nicholson, 1979) and brain (Raz & Hart, 1980) have been identified. Further degrees of specific localisation within the central nervous system have also been described (Brunson et al, 1978). Clearly these experiments could be interpreted as supporting the hypothesis that primary tumours consist of heterogeneous clones some of which have specific organ colonising properties.

One feature of the B16 melanoma model which is most difficult to understand is the fact that ten or more passages were required to develop target organ specificity to its maximum degree. This suggests that an alternative explanation could be that organ selective growth is

inducible by prior growth at that site. This latter explanation is somewhat unlikely in view of an experiment where B16 F1 cells were incorporated within small carrier beads which were injected intravenously and the cells allowed to form metastasis (Nicolson & Custed, 1982). After nine similar steps the resulting cells were re-injected without the carrier beads and found to have no greater cell affinity for lung than the original cell line. While making an inductive hypothesis less likely this does not really answer the original question concerning the number of passages required. Further evidence for lung specificity is seen when B16 F10 cells are injected into recipients which carry subcutaneous grafts of lung tissue. This results in the formation of tumour deposits in the graft (Hart & Fidler, 1980). This experiment also demonstrates the ability of B16 cells to survive passage through at least one capillary bed, before forming a tumour.

Although the F10 and F1 cell lines were derived from metastases, it can be shown that in a given sample only a few cells are able to generate new colonies when injected into animals (Hill et al, 1984). This was demonstrated by growing clones to different sizes; cells derived from small clones produced many fewer lesions when reinjected than the same number of cells from large clones. Using these methods it was shown that the difference in the F10 and F1 cell lines was related to differing rates of generation of metastatic variants. Elucidation of the precise developmental pathways involved in this process would perhaps help to clarify the mechanism by which repeated

passage led to the development of the site specific B16 cell lines.

Leaving aside the question of the relevance of the B16 model as a useful guide to behaviour of human tumours, it is clearly of interest to consider possible mechanisms at a cellular and molecular level for the phenomena described above. It would be reasonable to expect the differing properties of cell lines to be related to differences in their cell membranes. This was elegantly shown by the fusion of membrane vesicles produced by one cell line to viable cells of another line (Poste & Nicholson, 1980) which resulted in altered behaviour in vivo.

Attempts to demonstrate differences in membrane bound proteins by gel electrophoresis have not been very successful. Little difference has been shown between the proteins isolated from F1 and F10 cell lines (Nicholson et al, 1977, Warren et al, 1975). In the case of brain colonising lines increases were found in 90Kd and 100Kd glycoproteins as compared to the F1 cell line (Brunson et al, 1978). Raz and his co-workers (1980) have attempted to define membrane differences by a wide range of techniques. In this study high and low metastatic cell lines were similar with regard to patterns of cell surface carbohydrates although some differences were seen in lectin binding. Differences were also found in the level of 5' nucleosidase in the membrane, but study of membrane lipid composition showed no significant differences. Selection of cells for resistance to agglutination by wheatgerm agglutinin produced a much less metastatic cell line (Tao & Burger, 1977). Thus it can be concluded that despite the

demonstrable importance of membrane constituents in mediating differences in the metastatic behaviour of cells little progress has been made in understanding the molecular basis of this phenomenon.

A second approach to this question is to examine the adhesive properties and membrane-binding affinities of various cell lines. It was possible to isolate clones of Bl6 cells which had variable rates of detachment from plastic when incubated with EDTA (Briles & Kornfield, 1978). Cells which detached readily tended to form fewer tumours when injected intravenously. A similar result was seen with adhesion to a cellular substrate (Winkelhake & Nicolson, 1976). When adhesion to tissue sections was tested Fl0 cells were shown to have a markedly greater binding affinity for lung than other cell lines (Netland & Zetter, 1984). Other differences in <u>in vitro</u> cell adhesion have been described using platelets (Gasic et al, 1973) lymphocytes (Fidler & Bucana, 1977) and endothelial cells (Nicolson & Winkelhake, 1975).

In an attempt to further examine the problem of the relationship of the cell surface to <u>in vitro</u> and <u>in vivo</u> behaviour, monoclonal antibodies have been prepared against Bl6 cells (Vollmers & Birchmeier, 1983a) and selected for inhibition of plastic adhesion by the cells. Some of these antibodies appear to react specifically with a 40-50Kd antigen in Bl6 melanoma cells. These antibodies appear to have a significant effect in reducing metastasis in lung when cells were preincubated with antibody or the animal pretreated with antibody. These studies suggest that the behavioural changes may be the result of a subtle change in

the membrane perhaps related to one protein. However some caution is required in interpreting these experiments as the effects of the antibody could have been due to steric hindrance of in vivo adherence rather than the inhibition of the function of the bound protein.

In addition to these studies of the cell surface and cellular adhesion, behaviour has been correlated with a number of other phenotypic characteristics. Using fluorescent staining for DNA content and membrane constituents, it was possible to detect and isolate a metastatic B16 variant using a fluorescence activated cell sorter (Lessin et al, 1982). Other correlations with metastatic potential include the cAMP content of cells, (Shepard et al, 1984) resistance to anthracyclene antibiotics (Raz, 1982) and the effects of host immunity (Thomson et al, 1983). The possibility of a feedback type of growth stimulation in B16 cells involving insulin-like factors and growth hormone has recently been raised (Bajzer et al, 1984). It will be of interest to see whether differences in the level of activity of this pathway are found in other cell lines.

A final important property of the B16 system which has recently attracted considerable attention is the ability of sub-clones of cells to modulate the behaviour of other clones. The metastatic phenotypes of the B16 F1 and F10 are highly stable. This is seen by the widespread use of these lines by many investigators. Poste found that clones established from these lines were heterogeneous with respect to metastatic ability. The phenotype was highly unstable when further sub-clones were cultivated in vitro

(Poste et al, 1981). This instability could be corrected by mixing sub-clones together. Furthermore the use of drug sensitive and resistant clones showed instability could be re-introduced by application of a selection pressure by drug treatment. The conclusion of these studies was that the cells in the primary cultures tend to interact in such a way as to produce stability. This effect is not confined to B16 clones alone. Stabilisation can occur when B16 and K-1735 melanoma cells are mixed (Poste et al, 1984): normal cells and cells from other types of tumour do not do this. An analogous effect has been seen in vivo. Clones isolated from pulmonary metastases soon after injection have a similar metastatic potential when re-injected. This is not the case for clones obtained later which progressively diverge (Poste et al, 1982). This effect is enhanced in the case of spontaneous metastasis if the primary tumour is surgically excised (Poste & Grieg, 1982). As yet there is no information as to the mechanisms of these interactions. Clearly if this phenomenon is found to be a property of most malignant tumours it will have important implications for the treatment of cancer.

The various studies described above can be summarised as follows. After many years in culture B16 melanoma was found to be heterogeneous with respect to metastatic behaviour. In vivo selection yielded cells with specific organ homing properties. An important feature of this cell line may be its ability to generate new variants and this can be modulated in various ways. Despite extensive study there is little indication as to the molecular basis of these effects.

To place this work in context the answer to the following questions should be sought. Are the properties which have been described peculiar to B16 melanoma and to what extent can they be regarded as artefacts of tissue culture ?

c) Systems which resemble B16 melanoma.

The experimental model which most closely resembles the B16 melanoma is the K1735 mouse melanoma. This tumour arose in a C3H mouse following ultraviolet irradiation. A similar method was used to that which demonstrated clonal heterogeneity in the B16 melanoma (Fidler et al, 1981). Twenty of twenty-two clones derived from the K1735 tumour differed in metastatic potential from the parental line. The experiments were performed soon after the primary presentation of the tumour and have been used to counter the argument that the findings with B16 cells are an artefact of prolonged culture. Nevertheless the cells were grown in culture before the injection into the syngeneic hosts. Again following experiments performed with B16, K1735 induced lung nodules were used to derive new cell lines (Talmadge & Fidler, 1982). These were found to be more metastatic than the parent line following subcutaneous and intravenous injection and have been taken as supporting evidence of the origin of metastasis from distinct subpopulations of cells. As yet no attempt has been made to produce organ specific cell lines from K1735.

K1735 shares the obvious similarity with B16 of being melanocytic in origin. However tumours of other types have been studied in similar experiments. The UV-2237 fibrosarcoma line has shown heterogeneity (Raz et al, 1981)

after cloning as well as increased metastases by cells derived from lung lesions. This line has also been used in studies of clonal stability as described above for B16 and has given similar results (Cifone & Fidler, 1981). The 3LL lung carcinoma cell line after two passages through lung also showed greatly enhanced metastatic potential (Talmadge & Fidler, 1982). Thus it is reasonable to conclude that the type of heterogeneity demonstrated in B16 melanoma cells is not simply a peculiarity of this cell line since most of the essential features of the B16 model have been observed with other cell lines.

d) Systems which do not show heterogeneity.

It would be wrong to assume that the results of the experiments described above were universally applicable or indeed necessarily relevant to the behaviour of most tumours. Using a nickel-induced rhabdomyosarcoma it was found that cloned cell lines vary with respect to tumorogenicity and metastatic potential as compared to the parent line (Sweeney et al, 1982). However, it was found that clones which colonised the lung extensively following intravenous injection produced few metastasis after subcutaneous implantation and vice versa. This divergence of experimental and spontaneous metastasis has been well demonstrated by Tarin and Price (1979) using spontaneously arising mammary tumours (due to vertical transmission of murine mammary tumour virus). The cells were isolated and re-injected intravenously without prior culture. In about one third of tumours lung colonisation occurred, despite the fact that these tumours almost never metastasise in vivo. A similar result has been found with another non-

metastasising breast carcinoma (Williams et al, 1982). From these experiments it can be envisaged that while heterogeneity in potential to grow at a distant site may be present the cells with high colonising ability may lack the characteristics required to leave the primary tumour.

A second major objection to the general applicability of B16 melanoma and similar models is a possibility of culture induced artefacts. The lability of cells in vitro is a well known phenomenon. In the case of B16 cells clonal instability with regard to colonising potential has been demonstrated to occur and the composition of cellular populations can affect this process (Poste et al, 1981). In view of these objections a more meaningful experiment might involve spontaneous metastasis without in vitro passage of cells. This approach has been used to compare KHT osteosarcoma, B16 wild type cells, 3LL carcinoma and T241 carcinoma (Weiss et al, 1983). When minced metastatic tumour suspensions were used the KHT and B16 metastasis gave rise to more pulmonary lesions following subcutaneous injection than suspensions derived from primary tumours. However, the other cells lines did not show this effect. When excised fragments were used for implantation no significant difference was seen between the primary and secondary tumours. These authors also found that biopsy fragments from tumours of different ages and sizes did not vary in metastatic potential. These experiments do not support the idea that metastases arise from specialised populations of cells. However, one criticism of these experiments stems from the common observation that tumours

are morphologically heterogeneous and may consist of discrete zones of similar cells. It was found that the transplantation of small fragments resulted in much greater homogeneity of metastatic potential than the use of larger fragments (Fidler & Hart, 1981).

It will be apparent from the preceding discussion that a great deal of controversy and uncertainty surrounds the question as to whether or not the cells which form a tumour metastasis arise from a distinct subpopulation within the primary tumour. Given the doubts about repeated in vitro passage it is reasonable to conclude that the evidence in favour of the proposition is far from conclusive. It would be expected that cells derived from metastatic lesions without in vitro passage would give rise to increased numbers of metastasis. This has not been consistently demonstrated. There are however objections to this type of experiment as a valid test of the hypothesis. One of these takes the form that although highly metastatic subpopulations exist it is not necessary that an individual metastasis should arise from one of these. A further problem arises from the possible rapid generation of heterogeneity within the metastatic lesion itself. These possibilities are exceedingly difficult to test critically. These criticisms do not imply that this body of work should be discarded as a series of meaningless artefacts. Irrespective of whether they reproduce the situation in most tumours, model systems do offer considerable opportunity for the study of cellular mechanisms. An example of this is the organ homing capacity of various B16 cell lines. This represents a rare opportunity to study

cellular positioning at a molecular level and would have implications beyond the field of tumour biology. Clonal subpopulation interactions and dynamic heterogeneity are other phenomena of wide interest. It is perhaps unfortunate that these broader biological implications should have been to some extent neglected in the quest to prove or disprove the hypothesis which is described above.

e) Genetic aspects of the process of metastasis.

The process of metastasis is complex and a highly aberrant form of behaviour. It is therefore pertinent to ask what changes in gene expressions have occurred to allow this to happen. The most obvious approach to this problem would be to attempt to induce metastasising behaviour by transfection of cloned sequences from a cDNA library produced from metastatic tumours. This method has been used with some success to demonstrate the oncogenic effects of the ras family of oncogenes (Land et al, 1983, Gibbs et al, 1985). Recently DNA from a metastasising tumour was transfected into Ha-ras transformed 3T3 cells. This resulted in metastasis following subcutaneous injection, the metastatic lesions containing a common human sequence (Bernstein & Weinberg, 1985). As yet the sequences have not been characterised. The paucity of experimental data of this kind is an indication of the difficulties involved in obtaining and utilising appropriate tissues. The effect of the Ha-ras oncogene itself on the metastatic potential of cells has also been investigated (Greig et al, 1985). Transfectants of NIH 3T3 cells containing HA-ras sequences were found to be highly metastatic following subcutaneous injection. However surprisingly the 3T3 cells which were

used also produced tumours and metastasis albeit at a lower rate. This approach has been further developed (Muschell et al, 1985). These workers found that only the mutated or viral Harvey ras were effective in producing experimental metastasis. 3T3 cells in this study were non-tumorgenic. However these results were not repeatable in C127 transformed fibroblasts. This led to the conclusion that Harvey ras may act in concert with other cellular genes. It is clear that the study of genes involved in metastasis is only beginning and progress can be anticipated. The identification of the genetic basis of metastasis would rapidly lead to the solution of the problems of heterogeneity and metastasising subpopulations as discussed above.

In addition to the identification of specific genes involved in metastasis a second major question is the mechanism of genetic change which results in tumour progression. Again little is known about this subject. One intriguing aspect of it is the question of in vivo cell fusion as a generator of genetic change. There is some evidence to suggest that fusion of neoplastic and normal cells occurs in vivo. Human tumours grown in the hamster cheek pouch acquire hamster characteristics (Goldenberg et al, 1974). More definitively, tumours grown in bone marrow chimaeras undergo fusion with marrow derived cells (Weiner et al, 1972). In the B16 model using drug resistant markers no evidence of fusion with normal cells was found although fusion with other tumour cells seemed to occur (Hart, 1984). Following in vitro fusion, metastasising ability persisted when metastasising/non-metastasising

tumour cell hybrids were formed but not in metastasising/ normal cell hybrids. Suggestive evidence for a role for cell fusion in vivo in mediating progression was found using drug resistant markers and the karyotype studies in a variant of the MDAY-D2 murine tumour (Lagrade et al, 1983). Repeated sampling of the implanted primary tumour showed progressive loss of drug resistence markers and increasing mean numbers of chromosomes. Metastatic deposits derived from these tumours were found to consist of drug resistant hyperploid cells. Karyotypic studies suggested that these changes were the result of cell fusion.

Thus it seems likely that cell fusion may be one mechanism of tumour progression. Karyotypic studies of fused cells in turn may yield information concerning the genetic basis of metastasis.

f) The phenotype of the metastasising cell.

The preceding discussion focussed on the question as to whether specifically metastasising clones could be identified within a primary tumour. It is apparent that any cell which establishes a colony in a distant organ must have first completed a complex series of steps. These include escape from the primary site, entry into the circulation, interaction with blood constituents and the immune system followed by lodgement and growth at the distant site. It is equally apparent that failure to complete any of these steps will effectively prevent the formation of metastasis. Thus a complementary approach to the study of the cellular biology of metastasis is to dissect the phenotypic characteristics which allow cells to

behave in this way. Such characteristics form a diverse group and will be considered below.

g) Changes in cell to cell attachment in malignant cells.

The anatomical basis for many cell to cell attachments is well known. This takes the form of adhesion specialisations of the cytoskeleton such as desmosomes and the cytoplasmic interconnections seen in gap junctions (Hertzberg et al, 1981). Despite these detailed anatomical studies little is known about the regulation of expression of these characteristics. Another facet of the question of cell to cell attachment has been described by Edelman, (1983) in his description of cell adhesion molecules. These molecules show a degree of tissue and temporal variation which may be important in embryogenesis.

Cell attachments have also been studied in vitro. This work mainly employs the techniques of cell aggregation or alternatively assessment of strength of binding to various artificial substrates or other cells in culture. Notable results of this type of study may include the demonstration of differential cell adhesion and sorting where cells of various types are mixed together (Alberts et al, 1983).

Studies of the changes in the cellular attachment of malignant cells has been exclusively studied in vitro. This work stems from the observations of Coman (1953) which showed that the cells of malignant tumours were more easily detached. Since these early studies a very large volume of literature has been generated. Using B16 cells it was found that highly metastatic variants were less easily detached from plastic culture flasks by EDTA (Briles & Kornfield, 1978). In contrast no difference in EDTA

sensitivity was seen in fibrosarcoma cell lines but poorly metastatic cells were more easily detached by enzyme digestion (Varani et al, 1980a). A correlation was also found between attachment of this type of tumour cell to endothelial cultures and lung trapping after intravenous injection (Varani et al 1980b). These studies suggest that increased adhesiveness correlates with metastatic potential, but it is not a universal finding (Bubenick et al, 1976, Dorsey and Roth, 1973). The complex nature of this relationship has been illustrated by studies of the kinetics of aggregation when 3T3 cells and their SV40 transformed counterpart were compared: the relative adhesiveness depended on the shearing forces applied, the transformed cells being less adherent at low rates but more adherent at high rates (Elvin and Evans, 1982).

The molecular basis for many of these effects is not known with certainty. Most studies indicate the involvement of glycoproteins and glycolipids and in particular the carbohydrate moieties of these molecules. For example, inhibition of glycosylation in Bl6 cells by tunicamycin alters adhesion and reduces metastatic potential (Irimura et al, 1981). Alterations of lectin binding and metastasis in a rat mammary tumour is dependent on the expression of a high molecular weight glycoprotein (Steck & Nicholson, 1983) and alterations in the glycolipid GM3 have been seen in Bl6 sublines (Yogeeswara et al, 1978). Anti-cell surface antibodies have also been used to study this question. One series of monoclonal antibodies inhibits in vitro binding to plastic of a range of cell lines (Vollmers & Birchmeier, 1983b). Other monoclonal

antibodies prevent aggregation of hepatocytes by RAW117 lymphoma cells and inhibit liver metastasis (Nicholson, 1984).

It is apparent from this brief review that no definitive statement can be made about whether increased or decreased adhesion is a phenotypic correlate with metastasis. Both alternatives would be conceivable on purely theoretical grounds. All that can be said is that perhaps variation in some of the cell surface molecules which mediate in vitro adhesion is important in the malignant phenotype. There is no evidence that the particular molecules described play a part in the attachment of tumour cells to each other or to stromal elements in vivo. The main value of this type of experiment described above must be as a form of screening assay for detecting molecular events relevant to the expression of malignancy.

h) Relationship of metastasising cells to the basement membrane.

Basement membranes are a complex mixture of proteins and proteoglycans. The major protein components are the non-fibrillar Type IV collagen and laminin (Kefalides et al, 1979) the latter probably mediating the attachment of cells to the basement membrane (Terranova et al, 1980). The basement membranes separate epithelia, endothelia, nerve and muscle from surrounding connective tissues and appear to be synthesised by these cells (Kefalides et al, 1979). During the process of invasion and metastasis the tumour cells must penetrate a number of basement membranes both surrounding their tissue compartment and around blood

vessels. It is therefore relevant to ask whether the basement membrane surrounding tumour cells is defective and what mechanism tumour cells have for penetrating these structures.

Deficiency or poor formation of basement membrane is a common feature of malignant tumours. This can be demonstrated histologically, immunocytochemically or by electron microscopy. Studies which describe this as a feature of malignancy in breast and colon have been well documented (Siegal et al, 1981, Ozzella, 1959, Barsky et al, 1983 and Burtin et al, 1982). These findings could be due to either reduced synthesis of basement membrane or to dissolution by invading cells. Little information exists as to the former mechanism due to great difficulty in studying this in vivo.There is however some evidence to support the possibility that some tumour cells may degrade basement membrane. A distinct type of collagenase capable of digesting type IV collagen has been described (Salo et al, 1983). Correlation of the activity of this enzyme and tumour malignancy has been documented in a number of cases (Liotta et al, 1984, Starkey et al, 1984).

Attention has also focussed on the relationship between attachment to the basement membrane and malignancy. When B16 cells are selected for attachment to type IV collagen increased metastatic potential is found (Terranova et al, 1982). In this study in vitro binding to type IV collagen was mediated by laminin. In another study using B16 cells attachment to type IV collagen, invasion of amnion and pulmonary colonisation were enhanced by the addition of laminin but inhibited by fibronectin (Terranova

et al, 1984). A cell receptor for laminin has been purified from a breast carcinoma cell line (Malinoff & Wicha, 1983). The number of receptors present appear to be increased in some malignant tumours (Liotta, 1984). Moreover, blocking these receptors by degraded laminin appear to inhibit metastasis formation (Liotta et al, 1984). These studies indicate that cells may attach to type IV collagen through a receptor and laminin. This simple model does not apply to all systems which have been studied. The murine line MDAY-D2 has given rise to non-metastatic WBA^r mutants. This mutant avidly bind to type IV collagen but not the laminin. A similar pattern of binding with abrogation of metastatic properties may be produced by neuraminidase treatment of the parent line (Dennis et al, 1982). In summary, malignant cells have a complex relationship to basement membrane with degradation and increased binding being features of the malignant cell phenotype. In theoretical terms it is possible to envisage how both these features could promote metastasis formation. Increased basement membrane binding serves to localise the digestive action of cells; it could also be a factor in trapping at distant sites.

i) Cell Motility and Contact Inhibition of Movement.

The mechanism of movement of cells in culture has been intensively studied (Abercrombie, 1982). The main elements are the projection of lamellipodia and microspikes from the forward edge. Some of these attach to substrate others are swept backwards in the ruffling movement of the cell membrane. This process results in the attenuation of the rear of the cell which detaches and retracts into the cell

body. These movements are functions of the cytoskeleton particularly the ray of actin filaments which form complexes with various protein most notably myosin ATPase.

Changes in cell motility are an obvious feature of metastasising tumour cells. Such changes could either occur at the cytoskeletal level with acquisition of ability to move, or alternatively at the level of control of movement. The demonstration of enhanced motility in vitro has generally involved the use of two compartment systems analogous to the Boyden chamber. The boundary may either be an inert membrane or a piece of tissue. Using a simple porous membrane and radio-labelled cells it was found that malignant cells crossed the membrane whereas normal cells did not (Hart & Fidler, 1978). This type of experiment has also been performed using human amnion (Easty & Easty, 1974) and chick chorioallantoic membrane (Russo et al, 1982) as a barrier. Fidler and Hart used mouse bladder as a boundary and were able to recover and propogate a highly invasive subline of the B16 melanoma from cells which crossed the bladder wall (Poste et al, 1980, Hart, 1979). This is known as the B16 BL6 line.

The question of how these changes in motility are related to alterations in cytoskeletal function are at the present time obscure. As adhesion is an integral part of this process some of the work described above may be relevant to this question. An alternative approach is to consider the factors which inhibit the movement of normal cells away from their primary sites. Again this question has been exclusively studied in vitro. The key concept is inhibition of movement which results from the contacts

between cells. An *in vitro* model which was used to investigate this concept is one in which an explant of normal tissue confronts a culture of tumour or normal cells. Three possible outcomes of this experiment have been described (Abercrombie, 1979, Abercrombie & Heaysmann, 1976). When normal cells are used their motility ceases when they reach fibroblasts migrating out of the explant. When malignant cells are used they penetrate deeply into the sheet of outwardly migrating normal cells, whose spread may or may not be arrested by contact with the tumour cells. This type of analysis has been repeated using a wide range of tissues and cell lines (Marcel, 1983).

There can be little doubt that the findings described above are highly reproducible features of tumour cells in culture. However, it is equally true that the assay systems used are of a highly artificial nature and extrapolation to *in vivo* situations is not possible.

j) The relationship between invading tumours cells and surrounding connective tissues.

In order to metastasise tumour cells must have the ability to traverse surrounding connective tissue and enter lymphatic or blood vessels. In many human tumours it is apparent that tumour cells may have a profound effect on the connective tissues through which they are passing. Common examples include proliferation of fibroblasts and alterations in the components of the extracellular matrix, for example, collagen, elastin or proteoglycans and proliferation of endothelial cells to form new blood vessels. Similarly, when metastasising cells attempt to grow at a distant site the ability to stimulate the

connective tissue stroma would seem likely to be an important factor. The attempts which have been made to study these factors in experimental systems will now be considered.

Much attention has focussed on the production of degradative enzymes which allow motile tumour cells to traverse dense connective tissue matrix. These enzymes include the metalloproteinases such as classical collagenase and proteoglycanases. Collagenase acts on type I fibrillar collagen at one site which renders the peptides susceptible to further breakdown by other enzymes (Murphy & Reynold, 1985). Type I collagenase has been found in a number of tumours (Dresden et al, 1972, Dabbous et al, 1977, Robertson & Williams, 1969). It has been shown at least in some cases that the level of production of type I collagenase correlates with the ability to form experimental metastasis (Tarin et al, 1982). Plasminogen activator is another example of a neutral protease which is detectable in some tumour cells. However, there is considerable uncertainty concerning the correlation of this enzyme with metastatic potential (Tarin et al, 1982, Wang et al, 1980, Nicholson et al, 1976). Other enzymes studied include elastase (Jones & De Clerck, 1980) Cathepsin B (Sloan et al, 1982) and proteoglycanases (Nicholson, 1982).Most of the work cited above refer to cultured cell lines. To attempt to study enzyme activity in tissue homogenates would be extremely difficult in view of the diversity of reactive cells which are normally present in tumours.

Fibronectin is a high molecular weight glycoprotein

abundant in extracellular matrix and bound to cell surfaces (Yamada & Olden, 1978). Loss of this protein from transformed cells was one of the observations which led to its characterisation. Again there is some doubt as to the relevance of loss of fibronectin to metastasis with different results being obtained from various systems (Chen et al, 1976, Niemczuk et al, 1982).

Clearly there are distinctive parallels between these studies and the relationship of tumour cells to the basement membrane described above. However, as indicated the stromal reaction to tumour cells is much more complicated in vivo. Relatively little is known about the factors which could cause proliferation of connective tissue or changes in structure of the connective tissue matrix. In contrast the relationship of tumour cells to endothelial cells and blood vessels has been extensively studied. The importance of formation of new blood vessels to the process of metastasis has been demonstrated in a number of studies. The appearance of cells and cell aggregates in the venous effluent of an implanted tumour correlates with the formation and size of vessels in that tumour (Liotta et al, 1976). It was also found that the growth of tumour at distant sites depended on the growth of new vessels induced at that site rather than the reconnection of existing vessels (Folkman, 1975).

The formation of new blood vessels is a feature of the inflammatory response as well as the reaction to tumours. The process consists of the division of endothelial cells within existing vessels with penetration of the vascular basement membrane. The endothelial cells

then invade as cords which later form a lumen. The final phase involves formation of a basement membrane and association with pericytes (Schor & Schor, 1983). It is possible that during this maturation phase vessels are more permeable to invading tumour cells but this is difficult to test experimentally.

Considerable efforts have been devoted to characterise factors from tumours or other tissues which stimulate angiogenesis. The starting point for most of these studies is the development of a bio-assay system. Examples include a rabbit ear (Brem & Folkman, 1975) rabbit cornea (Gimbrone & Guillino, 1976) and hamster cheek pouch (Greenblatt & Schubic, 1968). A convenient and inexpensive method uses the chick chorioallantoic membrane (Auspuink & Folkman, 1977). Most of these techniques involve the implantation of tissue or purified material followed by observation of the growth of new vessels. An alternative method involves the use of isolated endothelial cells in vitro (Folkman et al, 1979). These studies have produced no shortage of angiogenic factors. A number of low molecular weight factors (less than 1000d) have been shown to produce angiogenesis in one or more of the systems described above. These include small copper binding molecules (McAuslan & Reilly, 1980) low molecular weight peptides (Feneslau & Wallis, 1982) and prostaglandins (Benezra, 1978).

Two high molecular weight substances should be particularly mentioned. The first is the 100Kd tumour angiogenesis factor first described by Folkman (1974). Its activity has been shown to reside in a group of heparin-

binding endothelial mitogens (Shing et al, 1984, Esch et al, 1985). The significance of these substances is suggested by the apparent association of mast cells with angiogenic tumours (Kesler et al, 1976) and by the inhibitory action of the heparin antagonist protamine sulphate (Taylor & Folkman, 1982). Apparently distinct from these substances is the recently cloned and purified 14 Kd protein known as angiogenin (Fett et al, 1985, Kurachi et al, 1985, Strydom, 1985). This substance does not bind heparin and is extremely active in the chorioallantoic membrane assay.

A third group of angiogenic factors are those related to coagulation. Fibrin degradation products are active in the chorioallantoic membrane assay (Thompson et al, 1985) and in addition, platelet derived growth factor is mitogenicfor endothelial cells in vitro (Seifert et al, 1984). The role of the coagulation system in metastasis will be further considered below.

The plethora of angiogenic factors and assay systems illustrate the complexity of the subject. However with progress in purification and sequencing of some of the factors some rationalisation will inevitably take place. It would be hoped that this would assist in answering a number of basic questions. Are multiple factors involved in the apparently stepwise process of angiogenesis? If so are tumour associated vessels defective as compared with those seen in a healing wound ? What contribution do tumour derived factors make to angiogenesis as opposed to those derived from inflammatory cells and coagulation factors ? As the answers to these questions may have

considerable therapeutic significance it is likely that they will be pursued with vigour.

It is likely that the considerable recent expansion of interest in tissue growth factors (Rozengurt, 1983) will result in greater understanding of the tumour-induced growth of connective tissue components other than blood vessels. This will give a much more complete explanation of the relationship of tumours to stroma and its effect on metastasis.

k) Circulation and Arrest of Tumour Cells.

Tumour cells which can detach from their primary tumour and traverse the surrounding stroma may gain access to lymphatics or veins and so enter the general circulation. The final destination of these cells will depend on haemodynamic factors and trapping of the cells in distant capillaries. Trapping in turn will be influenced by specific adhesion, the physical characteristics of the cell and extraneous factors such as the coagulation system.

Cytological examination of blood samples from mice inoculated subcutaneously with B16 melanoma and 3LL Lewis lung carcinoma showed that despite temporal fluctuations the carcinoma cells were able to enter the blood stream at a higher rate than B16 cells (Glaves, 1983). Although this was reflected in the development rate of metastasis, the number of circulating cells exceeded the final number of tumours by a very large margin. This and other studies (Butler & Gullino, 1975, Weiss, 1980) testified to the low efficiency with which circulating cells are able to generate metastasis. Clearly the majority of cells are injured or destroyed in the circulation. This could be

caused by the immune system or cells could sustain mechanical injury while in the circulation. With one cell line, passage through a capillary bed reduced the ability of cells to form tumours to almost zero (Sato & Suzuki, 1976). If the arrest of radiolabelled cells is measured little radioactivity remains in the body after a few days (Weston et al, 1974). Removal and culture of the lungs of animals after injection of tumour cells produces few viable tumour cells after 24 hours (Hewitt and Blake, 1975). Thus it appears that tumour cells are ill-adapted to the circulation and that this is important in limiting the development of metastasis.

The factors which may determine the growth of cells at specific sites are discussed above in relation to the B16 melanoma. However the tendency for a tumour cell to lodge in a capillary bed may be related to simple physical characteristics rather than the presence of receptors which mediate site-specific adhesion. In some systems neutralisation of charge either with cations (Hagemar, 1972) or by enzyme treatment (Proctor et al, 1976) increased the general tendency for spontaneous metastasis to form. However, this is not always the case (Sinha & Goldenberg, 1974). Another possible factor is the ability of tumour cells to deform in order to resist the destructive effect of shearing forces (Sato & Suzuki, 1976).

Aggregation is a further factor which could also influence the trapping of cells within the vasculature. The ability of Walker 256 carcinoma cells to form aggregates with platelets has been demonstrated (Warren, 1974).

Clearly such aggregates could effect trapping by virtue of size or by more specific adhesion perhaps to exposed basement membrane. This idea is corroborated by the decrease in metastatic lesions seen in thrombocytopenic animals (Gasic et al, 1968). In some but not all systems treatment with anti-platelet drugs reduces metastasis formation (Gasic et al, 1973). There is also some evidence that tumour cells differ in their interaction with platelets and that this may correlate with metastatic potential (Grignani et al, 1983). These observations suggest that platelets may influence metastasis formation in some types of tumour. Considerable attention has also been devoted to studying the role of fibrin formation in metastasis mainly by the use of anticoagulant drugs either to inhibit coagulation or to cause defibrinogenation. Some studies using Coumadins (Ryan et al, 1968) heparin (Suemasu and Ishikara, 1970) or snake venom (Wood & Hillgaard, 1973) have reported decreased metastasis whereas no effect was seen with other tumours (Hagemar, 1970). These results need to be interpreted with some caution in view of the multiple effects of some of the agents used.

In summary the stages of the metastatic process which involve local invasion and circulation of tumour cells are highly complex and poorly understood. However the studies described above serve to illustrate many of the non-specific determinants as well as the specific phenotypic features which affect the ability of cells to metastasise.

1) The host reaction to tumours.

A major theme in the experimental study of metastasis is the concept that host defences exist and that metastatic

cells may possess phenotypic characteristics which overcome such defences. The literature pertaining to this subject is vast and a comprehensive summary is not possible here. Three phenomena will however be considered. These are T lymphocyte reactions, natural killer cell reactions and cytotoxic macrophage reactions. All of these cells have effects in one or more model metastatic systems.

The T-lymphocyte response here will be taken to mean antigen-specific MHC class I restricted cytotoxicity. Evidence is lacking that the majority of tumours express novel antigens which are potential targets for a T cytotoxic response, but such antigens are found in some systems. The most commonly reported example is the polymorphic tumour-specific antigens of methylcholanthrene-induced fibrosarcomas and other tumours (Old et al, 1962). When tumour cells of this type are injected into syngeneic animals few tumours are seen, but the incidence is greatly increased if the host animal is immunosuppressed (Woodruff et al, 1984a, Woodruff et al, 1984b). In contrast, treatment with the T-cell suppressant cyclosporin A has little effect on epithelial tumours which are poorly immunogenic (Eccles et al, 1980). In the methylcholanthrene induced fibrosarcoma, the primary tumours may be removed after a short period of growth but metastasic deposits continue to present for long periods after surgery (Fisher et al, 1983, Gorelik et al, 1981). Combined treatment with specific tumour-related antigen and cyclophosphamide help prevent metastasis formation in this situation although neither are effective when given alone (Nomi et al, 1984). The Eb/Esb lymphoma model, also

chemically induced, has been shown to spontaneously generate highly metastatic variants which are resistant to *in vitro* lysis by cytotoxic T-cells reacting against a tumour associated transplantation antigen (Bosslet & Schimmacher, 1982). The fusion of Esb immunogenic cells with normal T-lymphocytes sometimes decreased immunogenicity and results in growth and metastasis in immunocompetent mice (Larizza et al, 1984). A third example which has been studied is a chemically induced rat mammary carcinoma. In this system immunisation with killed cells retards the growth of poorly metastasising tumours suggesting that they are immunogenic whereas this does not occur with highly metastatic types(Kim, 1970). Non-metastatic tumours of this type also evoke much greater degrees of reactive, lymphoid hyperplasia than more malignant variants (Yao et al, 1983).

These studies show that some tumours may be antigenic and that there may a good correlation between growth of metastasis and the absence of antigens capable of eliciting an immune response, although the precise details of response is seldom well-characterised. There is however, no definitive evidence that such mechanisms are frequently found in systems other than chemically induced tumours in rodents.

The second possible mechanism of host defence against metastasis is non-antigen dependent cytotoxicity, most notably in the form of natural killer cells (NK cells). NK cell activity is usually demonstrated by the cytotoxic action of spleen cells on a YAC-1 cell target without prior sensitisation (Gorelik & Haberman, 1981, Gorelik et al,

1984). This activity is sometimes divided into two types depending on the time of lysis of target cells, the prolonged reaction being described as natural cytotoxic activity (NC activity) (Stutman et al, 1978). These activities have a number of relatively constant characteristics. They increase with age (Keissling et al, 1975) and can be inhibited by treatment with cyclophosphamide (Mantovani et al, 1978) beta-oestradiol (Seaman et al, 1978) and antibodies against asiaolo GM1 (Kasia et al, 1981). The cells responsible appear to be a subset of the large granular lymphocyte seen in peripheral blood (Moore, 1985). These cells express the T8 (T suppressor/ cytotoxic) determinant and respond to interleukin II (Moore, 1985). Recently the beta T cell receptor gene has been shown to be rearranged in cells with NK activity (Yanagi et al, 1985). They have also some features of cells of the mononuclear phagocyte system (Moore, 1985). These features of differentiation, which hitherto seemed incompatible, are also seen in the T-gamma leukaemia described by Kadin (1981) which may be a tumour of NK cells.

A number of observations suggest that a non-antigen driven mechanism may in some systems be protective against the formation of metastasis. Nude mice which have minimal T-cell activity have significant NK activities which increase with age (Hanna, 1980). Metastasis in these animals is rare but can be induced by the agents described and metastasis can be abolished by transfusion with lymphoid cells bearing NK activity around the time of tumour cell inoculation (Hanna & Fidler, 1980). Finally, when UV2237

fibrosarcoma cells are co-cultivated with syngeneic spleen cells the survivors are more metastatic although they are identical to parent cell line in every respect other than NK cell binding (Hanna & Fidler, 1981). Similar results have also be obtained using clones derived from the 3LL tumour (Segal et al, 1982). In the context of the preceding discussions a number of observations concerning the recognition of tumour cells by NK cells are relevant. In a number of cell lines laminin binding appears to be correlated directly with sensitivity to NK cells (Hiserodt et al, 1985). Addition of laminin inhibited cell killing. The effect was specific for laminin and did not influence immunocytolysis. It has also been suggested that the antimetastatic effects of NK cells in vivo may be blocked by fibrin (Gorelik et al, 1984) as shown by an apparent NK cell anticoagulant synergism. These observations raise more general questions as to the mechanisms of NK cell recognition of tumour cells. Clonal analysis suggests that NK cells recognise only a few specificities unlike T-cells (Moore, 1985). Several suggestions as to a receptor have been made such as the glycolipid asialo GM2 (Young et al, 1981) and the transferrin receptor (Newman et al, 1984) but no consensus has been reached on this matter.

In addition to the antigen responsive lymphocytes and NK cells, macrophages represent a third possible mechanism of host lysis of tumour cells. Reactive macrophages are commonly found in human tumours. This may take various forms such as an infiltrate between neoplastic cells or sinus histiocytosis or granuloma formation in nearby lymph nodes. However, in comparison with the other cells

described above the role of macrophages in experimental tumours is less clearly defined. One of the difficulties in studying this problem is that macrophage activity is modulated by T-lymphocytes and that T-lymphocytes depend on macrophages for antigen recognition. It has also been suggested that macrophages may modulate NK cell activity (Djeu et al, 1979a, Djeu et al, 1979b).

Isolated macrophage populations have been shown to kill some types of tumour cell in vitro (Cohen et al, 1982). Poste et al (1979) have shown that treatment of animals with lung homing liposomes containing macrophage activators is effective in reducing the number of B16 lung metastasis. When the density of macrophages within lung metastasis was measured it was found to be highest in early lesions declining rapidly as the tumour grows (Bugelski et al, 1985). It has been suggested that this may be evidence for a tumour escape phenomenon.

It is apparent from this brief discussion that host cell responses to tumours have been clearly shown to prevent metastasis in some types of experimental tumours. However, it is far from established that these mechanisms have general applicability especially in human tumours. Attempts to identify tumour specific antigens in human tumours have not been very successful and in the absence of these it is difficult to make a case for antigen dependent immune responses. The evaluation of NK cell and macrophage functions will be greatly simplified when the recognition mechanisms of these cells are better understood.

m) Local regulation of growth.

The final stage in the metastatic process involves growth of cells at a distant site. Cells may reach a tissue, remain viable but fail to grow. This is the phenomenon of tumour dormancy which has already been described as it occurs in human tumours. Clearly this could involve either systemic repression of tumour growth or it could be confined to a particular site.

From experimental work a number of mechanisms have been postulated to account for this phenomenon. They can be divided into absence of growth factors (or presence of inhibitors) at a particular site or the growth inhibitory effect of the immune system.

Tissue growth factors are now being identified and characterised, but as yet there is little understanding of their precise physiological role. The involvement of such factors in the regulation of tumours however can perhaps be inferred by the effect of local operative trauma on the activation of dormant Walker 256 cells. (Fisher & Fisher, 1959). Similar activation occurs when oestrogen is administered to castrated animals bearing oestrogen dependent tumour cells (Noble & Hoover, 1975).

The possible effect of the immune system on tumour suppression can be demonstrated using animals from which a primary tumour has been excised. Activation of dormant cells can be achieved by the use of systemic immuno-suppression (Eccles & Alexander, 1975, Alexander, 1982) or by transplanting a lung to an immunosuppressed animal (Alexander, 1983). Cells induced to grow in this way are apparently similar to the original primary tumour (Eccles

et al, 1980). These experiments do not give an indication as to the mechanism by which the immune system is able to suppress growth and its relationship to other cytotoxic effects on human cells .

Another approach to this problem is to attempt to modify tumour growth by modifying the host tissue before induction of tumour. This is illustrated by the effect of local irradiation on the growth of metastasis at a given site, an effect which is unexplained but appears to be independent of immunological mechanisms (Peters et al, 1978, Milas et al, 1983, Wither & Milas, 1973). It could relate to post-injury regeneration or vascular changes. Irrespective of the mechanism, these experiments suggest the need for caution in the interpretation of experiments in which radiation is assumed to act as an immuno-suppressive agents in promoting tumour growth.

The preceding discussion is not an exhaustive account of tumour metastasis but serves to demonstrate the great complexity of this process. The remainder of this thesis will examine in greater detail some of the factors which regulate the growth of tumour cells at distant sites. Most of the work described will utilise experimental metastasis (intravenously injected tumour cells) to circumvent the complex problems of local invasion, angiogenesis etc. The questions to be considered include the role of the vasculature in determining the site at which a tumour cell will grow, and the effect which growth at a particular site has on the subsequent behaviour of tumour cells. In Chapter 3 the role which histocompatibility antigens may play in mediating this type of cellular interaction will be considered.

CHAPTER TWO

The effect of Intraperitoneal Passage on the behaviour of B16 melanoma and mouse salivary tumours cells.

CHAPTER 2

2.1. Introduction

This chapter will consider some of the factors which determine where a metastasising tumour will grow. The derivation and behaviour of the B16 melanoma cell lines has already been discussed. The F10 cell line was the result of 10 cycles of intravenous injection and passage of the resulting pulmonary metastasis. This produced a population of cells which is highly metastatic and selective for growth in the lung (Poste & Fidler, 1980). The F1 cell line is the result of one such passage and is poorly metastatic and less selective in its sites of growth. The behaviour and mode of derivation of the cells raises a number of important questions. Firstly, it is important to consider whether the cells used in these experiments show identical properties to those described in the original description of the B16 melanoma. This is important in view of the long period during which these cell lines have been maintained in culture. Secondly, is the characteristic behaviour of the F1 and the F10 cells a reflection of interactions with blood vessel endothelium or does it reflect differing abilities to grow within particular tissues ? Thirdly, as the F10 cells were derived by exposure to lung tissue is it possible to reverse the lung homing behaviour by exposing the cells to a different tissue environment? As the F1 cells are relatively unselected, can growth and passage at a particular site result in tissue trophism if the cells are not exposed to vascular endothelium ? Answers to these questions will be sought by examining the behaviour of F10 and F1 cells when grown in the peritoneal cavity.

The B16, F1 and F10 cells are derivatives of a malignant tumour some variants of which at least are able to spontaneously metastasise. To consider the question of the effects of distant sites on tumour growth it is of interest to consider a tumour which does not spontaneously metastasise. Such behaviour could be due to failure to invade the circulation by any of the steps in the metastatic process described in Chapter 1, but equally, it is possible that benign behaviour may be the result of inability to grow at a distant site. This possibility will be tested by direct transplantation of cell suspensions and fragments of a benign salivary gland tumour to a distant site thus excluding the effects of earlier steps in the metastatic process.

The correlation between cell adhesion and local invasion/ metastatic potential has been discussed in pages 32 and 33. Little, however, is known about the relationship between *in vitro* adhesion of tumour cells to a given tissue and the ability of cells to grow at that site. It has been shown that B16 F10 cells adhere more avidly to lung cryostat sections than to other tissues (Netland & Zetter, 1984), but no comparison was made with other B16 lines, a lymphoma cell line being used as the control. An attempt will therefore be made to directly compare the F10 and F1 cells in this type of assay and to observe whether changes in cell adhesion occur in parallel with changes due to *in vivo* passage. Allied to this study, the adhesiveness of the benign salivary tumour will be compared with its *in vivo* growth pattern.

MATERIALS AND METHODS

a) Cell culture methods.

1. Source of cells.

B16 F10 cells were obtained from the American type culture collection, Bethesda, Maryland. These were initially derived from the laboratory of Dr. I.J. Fidler (M.D. Anderson Hospital and Tumour Institute, Houston, Texas).

B16 F1 cells were kindly donated by Dr. C. Evans (Department of Experimental Pathology, University of St. Andrews). This line was also derived from Dr. Fidler's laboratory.

Both lines had been maintained in serial *in vitro* culture since their derivation about fifteen years ago.

2. Media

All experiments were conducted using the same media. Cells were grown in Hepes buffered Ham's F10 (Glasgow variant) (Gibco Ltd, Paisley, U.K.) containing 10% foetal calf serum, pH being adjusted using sodium bicarbonate solution. Other additives included Tryptose phosphate broth, L-glutamine and an antibiotic mixture (Penicillin G, Amphotericin B and Streptomycin). This medium was chosen after a number of preliminary experiments since it appeared to give the most rapid cell growth. It will henceforth be referred to as medium A (see Appendix 1).

When cells were being frozen for storage the same medium was used but with 20% foetal calf serum and glycerol (1:20 v/v) added as a cryoprotective agent (medium B) (see Appendix 1). When cells were being prepared for injection, final washing and resuspension was in minimum essential

medium (MEM) without the addition of foetal calf serum.

3. Storage and use of cells.

To ensure reproducible results a large frozen stock of cells was maintained. Cells were replaced from stock every two to three months.

Cells to be frozen were suspended at 4 - 5 x 10^6 cells/ml. in medium B. The vial was placed in a solid polystyrene block which was then left for five to six hours at -80°C before the vial was transferred to a storage unit. Long term storage was at -80°C. Stored cells were rapidly defrosted immediately centrifuged, washed and resuspended in medium A. Adherent cells were visible at 12 hours and growth was usually observable after 48 hours.

4. Handling of cultured cells.

Stock cultures were plated at 0.5 x 10^6 to 1 x 10^6 cells per 80cm^2 flask. A typical growth curve is shown in figure 2. Growth rates for both cell lines were relatively constant and predictable. Cells were removed from flasks as follows. Medium was removed and the growing surface was washed in Hepes saline (see Appendix 1). Five ml of 10^{-5}M. EDTA was added for one minute, most of which was then discarded. The flask was returned to the incubator for 2 - 3 minutes. It was then shaken vigorously for a few seconds until all the cells had been detached. Five ml of medium A was then added and cells were washed, counted and resuspended.

Cells for injection into animals were prepared as follows. Cultures were used 3 - 4 days after subculturing. These cultures were sub-confluent at this time. Preliminary experiments suggested that later cultures were sometimes less predictable in terms of in vivo behaviour. Only

adherent cells were used, detached cells and membrane vesicles (frequently present in the media) being discarded. All suspension for injection were used at a concentration of 106 cells ml-1.

5. Characterisation of B16 cell lines.

To ensure that cell lines were still melanocytic and not contaminated with fibroblasts following in vivo passage the following procedure was adopted. Cells were removed by EDTA as described and added to a Petri dish containing a sterile coverslip and 2-3 ml of medium A. After two days the coverslips were removed and fixed in Wolman's fixative for 30 seconds, then briefly washed in distilled water. Coverslips were then stained with haematoxylin and eosin or by Schmorl's method for melanin. To show evidence of melanin synthesis the dopa method was used. Coverslips were incubated in a solution of 1mg ml^{-1} of 3, 4 dihydroxy phenylalanine in phosphate buffered saline pH7.4 for 30 minutes. This solution was changed and incubation was continued for a further 2 hours at 37°C. As a control, coverslips were incubated with buffer alone. A positive reaction was seen by the presence of dense brown granules.

6. Comparison of in vitro growth of B16 cell lines.

Cell suspensions of B16 F10 and F1 and passaged F10 and F1 cell lines were added to 8 - 10 replicate 25 cm^2 flasks at a concentration of 2 x 10^5 per 5ml of medium. After 7 days, without change of medium, the cells were completely removed and counted. The cell lines were compared for cell yield using Student's t-test.

b. Conduct of animal experiments

1. Source of animals

All the experiments described in this chapter involving B16 cells used C57 BL6 mice: this is the syngeneic strain for the B16 melanoma. Breeding stocks of this strain were obtained from Olac Limited. Experimental animals were bred as follows. Ten brother/sister breeding pairs were maintained as a core stock. These were replaced by their own offspring as required. The progeny of this core stock were randomly mated for up to two generations.

The induction of salivary gland tumours was carried out using CFLP strain mice. These were derived from a colony which has been maintained for many years in Glasgow Royal Infirmary. These mice were not inbred.

2. Utilisation of animals.

Preliminary experiments with the B16 melanoma showed no difference between male and female animals with respect to tumour growth. Therefore both sexes were used, animals being randomly allocated to experimental groups. This was essential for reasons of economy and limitations on the size of animal colonies. In all experiments the animals used were 6 - 9 weeks of age. Again, this was the narrowest feasible age range. Breeding and experimental animals were housed at 21°C in a 12 hour light dark cycle. Water was freely available and the diet was the same throughout the study (CRM nuts K.K. Gruff, Croydon).

3. Injection techniques.

Intraperitoneal injections were performed using a 21g needle. In most cases 2 x 10^5 cells were injected in 0.2 ml of Minimal Essential Medium (MEM - Gibco Ltd).

Intravenous injection was performed using a 27g needle. The animal was immobilised in a specially designed holding cage. The tail was swabbed with xylene which produced venous dilatation within a few seconds. The needle was introduced into the tail vein freehand. It was readily possible to determine whether fluid was freely passing into the vein by resistance to the syringe plunger. Animals in which observable tissue swelling occurred were discarded. In animals used in experiments tumours at theinjection site were never seen. In preliminary experiments,the femoral vein was exposed and injected under general anaesthetic. However no advantage was found in using this method which was very time consuming. In most instances 0.2ml of cell suspension was injected. When groups of animals were being injected random samples of cells were smeared and stained to check for clumping but this never happened to a significant extent. In all cases more than 95% of injected cells were viable as determined by the trypan blue exclusion test.

4. Assessment of tumour growth.

i. Following intravenous injection.

The growth of tumours in lung was assessed 12 days after injection of cells in all experiments unless otherwise described. Two methods of assessment were initially compared. Lungs were removed and examined using x 40 magnification of a stereo microscope. All pleural or sub-pleural deposits visible at this power were counted. The same lungs were then fixed in formalin and paraffin embedded. Multiple levels were cut and the relative area of tumour measured by point counting using a semi-automated

system. (Northstar Advantage microcomputer with magnetic digitising tablet used in conjunction with a microscope and camera lucida). Fields and sections were counted in each case until the running mean fluctuated by less than 5%. A regression analysis was performed for the two methods which showed a close correlation (Fig. 3). The former method was chosen because of simplicity and similarity to the method used by most other workers with this model.

In addition animals were examined macroscopically and by random histological sampling for tumour deposits in other organs.

ii. Intraperitoneal injection.

Animals were examined at 5 days after injection. As both cell lines grew very rapidly at this site early sampling was required to prevent confluence of deposits and death of animals, which was almost 100% after 9 - 10 days.

Preliminary experiments showed that tumour deposits were most constant in the mesentery and visceral peritoneum of the small intestine. To quantitatively compare animals the small intestine was excised from the gastroduodenal junction to ileocaecal valve. The mesentery was then detached along its root. This specimen was cut into 1cm lengths and examined at x 40. All visible tumour deposits were counted.

Animals were routinely examined at post mortem and histologically sampled (particularly lungs). However, following intraperitoneal injection of cells distant metastatic deposits were never seen throughout the study.

5. Measurement of tumour cell trapping. (Appendix 2)
Cultures of B16 melanoma cells were prepared as above. Twenty four hours before use the medium was changed and replaced by medium A containing 3 MBq/100ml of Na ^{51}Cr (Amersham). The following day cells were removed and washed twice in MEM and then resuspended as before.

It was found that after 24 hours the cells were sufficiently labelled for convenient measurement. To examine the retention of ^{51}Cr replicate cultures were prepared and labelled as described above. After 24 hours the radioactive medium was removed and replaced by normal medium. Cultures were then harvested at various times and radioactivity counted in 2 x 10^5 cells using an LKB gamma counter with a 010-450KV window setting. The half life of the isotope retention was calculated from these measurements by fitting an exponential curve using regression analysis.

Animals were injected intravenously with 0.2 ml containing 2 x 10^5 radioactively labelled cells. Animals were killed 2 and 4 hours after injection. The lungs were removed, the heart was detached and the lungs were placed on filter paper to absorb excess blood. The lungs were then weighed and the radioactivity was measured by placing both lungs in a counting vial. The results were calculated as follows. The radioactivity per gram of tissue was calculated. To correct for the variable labelling of different cell samples this value was then divided by the radioactivity in 0.1ml of the cell suspension. The final result was multiplied by 1000 for convenience in analysis. In the following text all values referred to as cpm/g are

corrected in this way. Comparisons between groups of animals was made using Student's t-test.

c) 1. Serial transplantation of B16 melanoma cell lines.

B16 F1 and F10 cells were injected into the peritoneal cavity. The number of lesions arising were counted as described previously. The number of tumours produced by two cell lines were compared using Student's t-test.

In an attempt to modify the behaviour of these cell lines serial passage was performed through the peritoneal cavity. This was carried out as follows. Animals were injected by 2 x 10^5 cells and kept for 5 - 6 days. They were then killed and the abdomen was opened aseptically. A number of tumour deposits were then excised and placed in a sterile Petri dish containing MEM. The lesions were chopped and disrupted by repeated pipetting. After washing twice in MEM the cells were then re-injected into the peritoneal cavity of a new group of animals. This procedure was then repeated. The cells derived from these intra-abdominal passages were established as new lines in culture. Finely chopped disaggregated fragments were placed in 25cm^2 flasks together with 5ml of the medium A. After 24 hours the medium was changed to remove debris and the cells left to grow.

In the case of later passages of F1 cells, increased numbers of cells and longer incubation periods were required (see Results section below).

2. Assessment of the effects of passage.

Cultured cells derived as above from various passages of both F1 and F10 cells were injected intravenously and intraperitoneally into groups of C57 BL6 mice. The methods

used were identical to those described in pages 59 and 60. The number of tumour deposits arising was compared with the original cell lines using Student's t-test. The use of cultured rather than freshly isolated cells was essential here to ensure a single cell suspension and freedom from toxic cellular debris before intravenous injection.

The trapping of F1 cells passaged through the peritoneal cavity 16 times was assessed as described above using ^{51}Cr labelled cells. The degree of lung trapping was compared with unpassaged F1 cells using Student's t-test.

d) 1. Induction of salivary tumours.

Tumours were induced using mouse polyoma virus which was a gift from Dr. P. Lamey, Glasgow Dental Hospital. The virus was prepared at a concentration of 10^9 plaque forming units (pfu) per ml.

After preliminary experiments it was found that CFLP mice formed tumours at the highest frequency and these were used in the remainder of experiment. Newborn mice (within 24 hours of birth) were subcutaneously injected in the neck region with 25 µl of virus suspension and then returned to their mother. The animals were weaned at six weeks and observed for evidence of tumour development.

2. Transplantation experiments.

The aim of these experiments was to examine whether salivary tumours grew at a distant site following transplantation. As the CLFP strain is not inbred it was decided that all transplantation procedures using these mice should be performed as autografts. Tumours were excised as follows: Tumour bearing mice were anaesthetised with ether. A midline incision was made over the tumour

and the skin reflected, the tumour was mobilised by blunt dissection and as much removed as possible without causing excessive haemorrhage. When haemostasis was secured the wound was closed using black silk sutures.

3. Preparation of cell suspensions from Salivary Tumours.

Tumours excised as above were placed in a sterile Petri dish containing a small volume of MEM. The tumour was finely diced using two pairs of sterile scissors. The larger fragments were then removed and the remainder aspirated into a syringe and forced through a 19g needle several times to further disaggregate the cells. The resulting suspension was centrifuged and washed twice in fresh MEM. Viability of the cells were checked by 1% trypan blue exclusion and a final suspension of 5×10^6 cells ml^{-1} prepared.

Fragments of tumour were prepared for transplantation by chopping the tumour with scissors and selecting fragments of approximately $1mm^3$ in size.

Cell suspensions were injected directly through the abdominal wall of the animal from which they were obtained.Fragments were transplanted into the peritoneal cavity through a small incision made under general anaesthesia. This was closed with a single silk suture.

Animals were kept for a minimum of 4 weeks. They were then killed and thoroughly dissected. The peritoneal cavity was inspected under a dissecting microscope for evidence of tumour deposits. Lesions found were sampled histologically.

e) Assessment of in vitro cell adhesion.

1. B16 cells.

Cryostat sections were used as substrates to test the relative adhesiveness of passaged and unpassaged F10 and F1 cells. Five µm sections were cut from C57 BL6 liver, lung and F10 and F1 tumour masses grown in the peritoneal cavity. Pieces of parietal peritoneum were excised and rolled around a sliver of wood. This was snap-frozen and sections cut tangentially.

Cell suspensions were prepared as described above. The cryostat sections were air-dried and 200µl of cell suspension (106/ml) added to cover each section. After incubation at 4°C for 2 hours the sections were washed for 5 minutes in tris buffered saline, fixed in Wolman's fixative and stained with H & E.

The area of each section was measured using the same semi-automated morphometry system as described on page . The number of cells adhering to the sections were counted and expressed as cells per mm2. Holes in the section were subtracted from the area and cells not adherent directly to the tissue were not counted. Five sections were measured for each tissue with each cell type. A few sections in which large numbers of cells were adherent to the glass slide surrounding the section were excluded. The final result was expressed as cells adhering to lung, liver or peritoneum divided by the average number adhering to the section of autologous tumour.

2. Polyoma salivary tumour cells.

The protocol used was essentially similar to that described above. As the tumour cells could not be

successfully cultured, freshly isolated cells were used. Cryostat sections of liver, kidney, normal salivary gland and the peritoneum were prepared from the same animal as the tumour cells. Three sections of each substrate were measured for ach animal. The results were calculated as for the B16 cells.

2.3. RESULTS

a) Comparison of the metastatic potential of B16 F10 and F1 cells following intravenous and intraperitoneal injection.

1. Intravenous Injection.

A pilot experiment was conducted using B16 F10 cells. The number of tumour deposits on the pleural surface of both lungs was compared with the number of tumours per square mm on histological sections (Fig. 3). The correlation coefficient obtained ($r^2 = 0.78$) led to the conclusion that counting surface deposits was a satisfactory method and this practice was subsequently followed.

Twelve days after injection with 2×10^5 B16 F10 cells animals were killed and the tumour deposits in the lung counted. The mean number of tumours was 212.19 ± 23.8 (mean $\pm$ 1 standard error) from 32 animals. These animals were further examined at post mortem and no other tumours were found. In a comparable experiment with B16 F1 cells a mean of 5.0 ± 1.9 (1SE) tumours per animal was found from 10 animals (Fig. 4). In some animals injected with F1 cells an occasional tumour was found in the liver. There was a significant difference in the lung colonising capacity of F1 and F10 cells ($p < 0.0001$).

Intraperitoneal injection.

B16 F1 and F10 cells were injected into the peritoneal cavity and the number of deposits on the small intestinal mesentery counted after 5 days. With B16 F10 cells a mean of 66.5 ± 5.1 (1SE) deposits were found in 15 animals. B16 F1 cells produced a mean of 76.7 ± 11.08 in 15 animals (Fig. 5).

When these means were compared there was found to be no significant difference in the ability of these cells to grow in the peritoneal cavity of the C57 BL6 mouse. There was no evidence of distant metastasis with either cell line.

b) i. Effects of intraperitoneal passage on B16 F10 cells.

The ability of cells to form intra-abdominal tumours was assessed following 12 and 18 passages through the abdominal cavity. After 12 passages the mean number of tumours per animal was 69.5 ± 6.32 (ISE) (N =19). After 18 passages the number of tumours was 103.27 ± 7.78 (1SE) (N =11). After 18 passages the number of tumours was significantly greater ($p < 0.0001$) than obtained with unpassaged cells 66.5 ± 5.1 (1SE) (N = 15).

Cells derived from the 18th passage were injected intravenously and the number of lung lesions compared with the unpassaged F10 cells. The average number of tumours per animal was 260.07 (N = 17) as compared with 212.19 ± 23.8 (1SE) (N = 32) for unpassaged B16 F10 cells (Fig. 6). This difference was not significant. Animals injected with passaged cells were carefully examined and no tumour was found in any animal tissue except in the lung.

ii. Effect of repeated intraperitoneal passage on the behaviour of B16 F1 cells.

The ability to form intraperitoneal tumours was assessed after 9 and 16 passages. After 9 passages a mean of 10.8 ± 1.93 (1SE) tumours per animal was seen (N = 5). After 16 passages a mean of 11.13 ± 1.74 (N = 12) tumours per animal were found. Cells from both passaged lines formed significantly fewer ($p < 0.01$) abdominal lesions than

unpassaged F1 cells (76.7 ± 11.08, N =15) (Fig. 7).

Cells from these passages were also tested by IV injection. After 9 passages the mean number of tumours formed in the lung was 2.0 ± 0.18 (N = 6). This was not significantly different from the unpassaged cells (5 ± 1.9. N =10). However after 16 passages the numbers of lung lesions greatly increased to 66.89 ± 11.91 (1SE) (N =11). This was significantly greater than the unpassaged cells ($p < 0.001$). Tumours were not seen in any organ other than the lungs in animals injected with F1 cells passaged 16 times through the peritoneum.

The intraperitoneal passage of F1 cells proved to be rather more difficult than the passage of F10 cells. On two occasions cells failed to grow at 6th and 8th passage respectively both in vitro and when injected intraperitoneally. Continuance of the passage on the third occasion was only achieved by increasing the cell concentrations after the 6th passage and allowing the tumour to grow for more than 2 weeks.

On each of these three occasions unpassaged F1 cells were used to begin a new series of passages. Unfortunately, no stored cells were available from the first two series of passages.

c) Trapping of ^{51}Cr B16 F10 and F1 cells following intravenous injection.

i. Retention of ^{51}Cr in cells in vitro.

The radio-activity remaining in replicate cultures at varying times after removal of radioactive medium was measured. From this data it was estimated that the half life of retention was about 12.5 hours (Fig. 8).

2. Comparison of lung trapping of F1 and F10 cells.

The radioactivity of the lungs was measured in groups of animals killed at 2 and 4 hours after injection of radiolabelled cells. At 2 hours the mean radioactivity after injection of F10 cells was 3356 ± 352 (N = 18) $cpmg^{-1}$ and after injection of F1 cells was 1260 ± 200 (N = 16) $cpmg^{-1}$. This difference is significant at ($p<0.0001$) (Fig. 9). At 4 hours much lower levels of radioactivity were observed. In the case of F10 cells the mean was 403 ± 74 (N =16) $cpmg^{-1}$ and F1 cells 209 ± 34 (N = 7) $cpmg^{-1}$ (Fig. 10). This again is significantly different ($p< 0.05$).

The trapping in lung of radiolabelled F1 cells was compared with radiolabelled F1 cells following the 16th intraperitoneal passage (F1 P16). The mean lung radioactivity after 2 hours following injection of 16th passaged cells was 1511 ± 250 (N = 7) $cpmg^{-1}$. This is not significantly different from the unpassaged F1 cells (1260 ± 200 $cpmg^{-1}$).

d) The effect of intraperitonal passage on in vitro growth.

The yield of cells after 7 days in culture was measured for B16 F10 cells, F1 cells, F10 cells derived from passage 18 (F10 P18) and F1 cells from passage 16 (F1 P16). F10 cells produced (2.38 ± 0.7) x 10^6 cells/$25cm^2$ flask, F1 cells produced (2.18 ± 0.9) x 10^6 cells/$25cm^2$ amd F10 P18 produced (2.24 ± 0.05) x 10^6 cells/$25cm^2$. There was no significant difference between these cell lines. On the other hand the F1 P16 line produced only (0.1 ± 0.01) x 10^6 cell/$25cm^2$. This was significantly less ($p<0.001$) than any of the other cell lines. This result confirmed the great difficulty in propagating passaged F1 cells.

e) In vitro cell adhesion of B16 cells to various organs.

The adhesion ratio for F1 F10 F1 P16 and F10 P18 cells were calculated by dividing the adherent cells per mm^2 on liver, lung and peritoneum by the number adhering to a section of the same tumour. These results are shown in table 1 below:

Table 1

	Lung	Liver	Peritoneum
F1	1.02 ± .3	0.25 ± .03	1.03 ± 0.02
F10	0.65 ± .1	2.25 ± 1.1	1.05 ± 0.5
F1 P16	0.65 ± .1	1.39 ± 0.5	0.25 ± .01
F10 P18	0.39 ± .02	0.16 ± 0.6	0.5 + .17

There is clearly no significant correlation between the cell adhesion assessed by this assay and the pattern of in vivo growth which was observed after intravenous or intraperitoneal injection.

Figure 11 shows an examples of one of these preparations.

f) Morphological studies of B16.

1. Cultured cells.

B16 F1 and F10 and passage cell lines had an essentially similar appearance in culture. All cell lines grew mainly in the form of spindle shaped cells (Fig. 12). An exception to this was seen in the 8th and 9th passages of the F1 cells. At this stage the cells had a more epithelioid appearance and frequent binucleate cells were seen (Fig. 13). These changes disappeared in later passages the cells reverting to a more spindle shaped appearance. At low cell density some cells showed extensive spreading with long cellular processes leading to

an almost neuronal appearance. A feature of all the cell lines in vitro was the release of membrane bound vesicles into the medium. These were readily visible with the light microscope. At higher cell densities both cell lines behaved as transformed cells with absence of contact inhibition readily apparent.

Passaged cells and their parent cell lines all stained positively with Schmorl's method and the vast majority of cells showed evidence of melanin synthesis by the DOPA staining method (Fig. 14). Many cells were also pigmented and release of melanin into the medium was readily apparent after a few days in culture.

Cells prepared for injection showed no tendency to aggregate.

2. Metastatic lesions.

Pulmonary lesions were identical irrespective of the cell of origin. The lesions contained a relatively uniform population of large epithelioid type melanocytes often with prominent pigmentation. Nuclei were large and moderately pleomorphic with prominent nucleoli. Metastatic lesions within the lung were frequently angiocentric (Fig. 15) although tumour cells were not often seen within blood vessels. Tumour deposits on the lung surface were often composed of relatively flat plaques of cells apparently spreading along the pleural surface (Fig. 16). Tumour cells did not invade across the pleural surface and pleural adhesions were never seen. The lesions which were produced in the abdominal cavity were identical to those seen in the lung. Macroscopically two distinctive type of lesion were seen. An almost invariable feature of animals injected with

tumour cells into the peritoneal cavity was the presence of a large friable mass of tumour in the left hypochondrial region related to the spleen and stomach. This was loosely attached to the surrounding structures and was readily removed for use as the the source of cells in passage experiments. Histologically these lesions sometimes showed areas of necrosis but blood vessels also were a prominent feature presumably resulting from tumour-stimulated angio-genesis. The second type of lesion took the form of small deposit which were present mainly in the mesentery of the small intestine and associated with the intestinal serosa (Fig. 17, 18). The lesions were comparable in size to those seen in the lung. Although the tumour deposits were firmly attached they were not invasive or destructive of surrounding structures. Tumour deposits on the parietal peritoneum were rare in comparison to those on visceral surfaces.

g) Characteristics of Mouse Salivary Tumour.

All the tumours examined were identical in appearance. They were multilobed, encapsulated and consisted of regular polyhedral cells with relatively little nuclear abnormality. Mitoses were frequent, reflecting the rapid growth but no abnormal mitotic figures were seen. The tumours were sometimes seen to form small acini containing PAS positive material particularly at the edge of the nodules. A complete fibrous capsule was present and local invasion was never seen (Fig. 19). In some cases a lymphoplasmacytic infiltrate was present. In over 100 animals examined no evidence of metastasis was seen.

The median time when tumours became visible was 16 weeks after injection of virus. Tumours ultimately occurred in 32% of animals (2:1 male:female).

h) Transplantation Experiments using the Mouse Salivary Gland Tumour.

When cell suspensions were prepared from these tumours and injected subcutaneously or into the peritoneal cavity no tumours were seen after one month (N = 10). In this period residual tumours in the neck had usually regrown to a large size.

When small cubes of tumour were placed in the peritoneal cavity many were firmly attached and viable after one month but showed little evidence of significant growth compared with the rate of regrowth of the primary tumour. Histological studies of these lesions showed them to be attached to the peritoneum by fibrous tissue and were often related to the healed laparotomy scar (Fig. 20).

i) In vitro adhesion of Mouse Salivary Tumour Cells.

Adhesion of polyoma salivary tumour cells is expressed as a ratio of cells per mm^2 attached to the substrate tissue as compared to cells per mm^2 attached to a section of tumour from the same animal. The results were as follows:

Table 2

Kidney	0.72 ± 0.19	N = 8
Liver	0.65 ± 0.15	N = 8
Peritoneum	1.4 ± 0.57	N = 6
Salivary Gland	2.4 ± 1.3	N = 9

Using a paired t-test adhesion to liver and kidney was significantly less ($p < 0.01$) than to autologous tumour. However there was no significant difference between tumour, salivary gland and peritoneum.

2.4. DISCUSSION

The B16 F10 and F1 cells which were obtained for this study were remote from their original source; about 20 years having elapsed since they were derived from a mouse tumour. It was therefore essential that the characteristics should be verified and compared with their original description (Poste and Fidler 1980). Morphological studies of both the F10 and F1 cells clearly showed them to be melanocytic in differentiation. The medium used in this study (enriched Ham's F10) differed from that used by other investigators. It was chosen and formulated from pilot experiments which indicated that it gave clearly superior cell growth. The assessment of metastatic colonisation of the lung was identical to that used by most other investigators (Fidler & Kripke, 1977, Poste & Nicolson, 1980). It was compared with a morphometric method but in view of the close correlation the latter was abandoned because of the effort involved was much greater than counting pleural lesions. The number of lung tumours produced by the two cell lines was similar to that described by others although the F1 cells were perhaps rather less metastatic. Lesions outwith the lungs were occasionally found after injection of F1 cells but never with F10 cells.

It has previously been suggested that the difference in lung homing potential of the F1 and F10 cells may be due to the initial pulmonary trapping following intravenous injection (Fidler, 1975). This experiment was repeated using ^{51}Cr instead of ^{125}I-iododeoxuridine.Chromium labelling is a much simpler procedure but has the

disadvantage that the label is gradually lost, being released from cells by passive exocytosis. However chromium has been used successfully for this type of experiment by other investigators (Suemasa and Ishikara, 1970) F1 and F10 cells showed no difference in labelling characteristics and the rate of loss of label as determined over 24 hours suggested that the loss would not materially affect trapping measurements made within four hours. It was found that F10 cells were more avidly trapped in the lung than F1 cells both at 2 hours and 4 hours after intravenous injection. However the overall radioactivity was much less at 4 hours for both types of cells. Part of this loss may be due to the loss of label from trapped cells but from the data presented this probably does not account for the whole of the reduction. The residue is likely to represent cell loss from the lung either by passing through the capillary bed or by traumatic lysis. The latter phenomenon has been described in other systems (Sato & Suzuki, 1976). It is concluded that only a relatively small proportion of the cells which initially lodge in the pulmonary capillary bed are likely to emigrate into the parenchyma and form nodules of tumour. In this respect the B16 cell lines used in the present study resemble those described elsewhere.

When injected intravenously the F10 cell line is highly specific for lung whereas the F1 cell line has a much lower tendency to grow there. In preliminary experiments it was found that both cell lines would grow readily in a variety of sites when injected directly; the peritoneal cavity, pleural cavity and subcutaneous tissues

were tested. It was therefore decided to attempt to compare these two cell lines quantitatively with respect to local growth. Such comparison has not been previously reported. The peritoneal cavity was thought to have a number of advantages for making this comparison. Firstly cells would be free in suspension and in order to grow they would require to attach to a surface. Thus, tumour deposits formed in the peritoneal cavity could be assumed to be formed from single cells and in this respect would be more comparable with the results of intravenous injection. It should be noted that cells injected intraperitoneally or intravenously were single cell suspensions, with no evidence of cell aggregates as far as could be determined by light microscopy. Direct injection into a solid organ such as liver or skin would have resulted in a compact mass of cells and no indication would have been given as to the number of initial cells actually capable of growth. Secondly, the small intestinal mesentery was a readily identifiable structure in which to count the number of deposits. When F1 and F10 cells were compared in this way no difference was found in the formation of metastatic deposits in the small intestinal mesentery. The finding that the F1 and F10 cells were equally tumourogenic following direct injection into the peritoneal cavity is consistent with the results of trapping studies which suggest that the difference in lung homing behaviour of these cell lines may be at the level of interaction with the pulmonary vascular bed. However, since it was not possible to test tumour forming ability after direct intrapulmonary injection it must remain

possible that the environment of the lung could result in the growth differences between the cell lines. Nevertheless, both F1 and F10 are able to grow at a number of sites (skin, peritoneum, pleura) where metastasis are never seen after IV injection which again suggests that the site specificity at least in part may be a function of cell interaction with the vascular system. The ability of F10 cells to form deposits in subcutaneous pulmonary grafts (Hart & Fidler, 1980) and relative lung specificity after intra-arterial injection (Weiss et al, 1984) suggest that at least some cells capable to forming tumours survive as least one passage through a capillary bed.

The explanation given for the lung specificity of the B16 F10 cell line is that it is a result of a selection of lung homing cells from an initially heterogeneous population by repeated passage. In view of the results obtained by intraperitoneal injection, it is relevant to ask whether repeated passage of cells from mesenteric F10 tumours following direct injection will result in a cell line with increased tumour forming ability at this site, and if such a line can be derived, whether it will form tumours in the peritoneum when injected intravenously. When F10 cells are repeatedly passaged through the peritoneal cavity it was found that the number of mesenteric tumours following IP injection increased significantly by about 50% at passage number 18. However, the number of lung tumours which resulted from IV injection of these cells was not significantly different from the unpassaged cells, and no tumours were seen outwith the lungs.

These results show that the cell line produced by repeated intraperitoneal passage (F10 P18) has an increased ability to form tumours in the peritoneal cavity after direct injection, but has retained the parent cell lines tumour forming properties after IV injection. This excludes the possibility that the mesenteric deposits are a result of a discrete subpopulation of cells distinct from those which form lung tumours. However it suggests that the two properties are independent. Increase in mesenteric tumour formation, without change in lung tumour forming ability, could have resulted from selection of a population with enhanced mesenteric tumour forming ability from a larger population with constant lung colonising potential. Alternatively, the increase in mesenteric tumour formation may have been induced by the environment of the peritoneal cavity. The possibility of induction of lung homing was tested using F10 cells encapsulated in microspheres, thus eliminating specific lung trapping (Nicolson & Custed, 1982). No such effect was found. Nevertheless, this does not exclude the possibility of an inductive effect causing increased mesenteric tumours after direct injection. Avoidance of the circulation may have been expected to increase the efficiency of the process of tumour formation but even after IP injection less than 1:1000 cells appear to give rise to tumours. This observed inefficiency is consistent with the dynamic heterogeneity model which postulates that tumour forming variants are generated at a low rate from non-tumour forming cells (Hill et al, 1984). The increased generation of these variants by repeated passage could be due to either selection or induction.

A selective mechanism would be possible if colonies of tumour cells at a particular site showed a slightly greater tendency to generate variants capable of forming tumours at that site. This could account for stepwise evolution of population with increased tumour forming potential.

Repeated intraperitoneal passage had a much more profound effect on the F1 cells. Two distinct phases can be discerned. After 8 - 9 passages the resulting cells showed distinct morphological changes, becoming more epithelioid in character and more often multinucleate. This was associated with a very marked decrease in the ability of these cells to form tumours either after intraperitoneal or intravenous injection. Indeed on two occasions the passage was terminated at this stage. Continuation beyond this stage was only possible in a third experiment by increasing the number of cells injected in later passages. At the 8th and 9th passage it was almost impossible to propagate these cells in vitro.

During the later passages of the third series the cells once more began to resemble the unpassaged F1 cells morphologically. Cell growth and growth in the mesentery following intra-peritoneal injection remained significantly less than the unpassaged cells. However surprisingly the lung tumour forming ability of the passaged cells increased to a relatively high level (about 30% of that seen in the F10 cells). This increase in lung tumour formation could not be correlated with any increase in the trapping of radio-labelled cells. As described above the trapping of F1 cells was almost half that of F10 cells although the ratio

of tumours was about 1:20. Therefore it is apparent that lung trapping is not the sole limiting factor which determines the rate of lung metastasis of F1 cells. It would appear that F1 P16 cells had acquired an increased capacity for growth at the same level of trapping.

The effect of intraperitoneal passage on the F1 cells was unexpected and clearly contrasts with the effect on the F10 cells. The initial inhibition of growth and tumour forming ability would be most compatible with an inductive effect of the peritoneal cavity. As the unpassaged F1 cell line contained a large number of cells able to form mesenteric tumours, it is difficult to envisage how these could be removed by selection. Moreover, it is difficult to see how an in vivo selective pressure could result in a population of cell which grow poorly in vitro. The emergence of a cell line with a relatively high lung colonising ability is also difficult to explain. One possibility is that because of poor in vitro growth the later passages were oligoclonal in culture. This could have led to instability in lung colonising ability as has been previously described (Poste et al, 1981). Therefore the increased lung colonising ability of the F1P16 cells could be an indirect consequence rather than a direct result of intraperitoneal passage.

The intravenous injection of cells which have been repeatedly passaged in the peritoneal cavity did not result in tumours in the peritoneum (or any other organ other than the lungs). It could be argued from this result that intravenous passage and contact with the vasculature is essential for the selection of site specific variants such

as the F10 line. However as the results described above show this is not the sole possible mechanism: lung growing F1 cells arose without prior contact with the lungs (since the derivation of the cell line).

The above discussion has centred on the B16 melanoma cell line which was derived from a malignant tumour. However it is relevant at this point to consider the situation in a benign tumour. The polyoma induced tumour used in these experiments never metastasises and has the morphological characteristics of a benign tumour. The reason for the benign behaviour of this tumour could be failure to complete any of the complex steps in the metastatic process which are described above in Chapter 1. Alternatively, the cells may be incapable of growth in vivo at any site other than the salivary gland. To test the latter possibility, cells at very high concentration were injected directly into the peritoneal cavity. Despite the fact that the primary tumours were rapidly growing no growth was observed in the peritoneal cavity even after one month. This was also the case for subcutaneous injections. However, when small fragments of tumour were transplanted into the peritoneal cavity these remained viable, often attaching to the laparotomy scar but without visible growth. These experiments were performed as autografts and frequently the primary tumour had regrown to a large size during the experiment. These experiments lend further credence to the idea that even after direct injection some tissue environments do not permit the growth of some types of tumour cell. It is possible that the failure of cells in suspension to grow was due to damage

during isolation. It is difficult to formally exclude this possibility, but the methods used are widely employed in this type of application and the number of cells used was very high, making it likely that some competent cells would be present. Therefore it is reasonable to assume this to be an unlikely explanation.

A problem with the polyoma salivary tumour which prevented further exploitation of this model is that the animals used were not inbred so experiments had to be performed as autografts. Pilot experiments with various inbred strains produced poor yield of tumour. This problem was further compounded by the failure of attempts to grow the tumour in vitro.

It was hoped that the in vitro adhesiveness of tumour cells to various tissues would provide a simple screening method of predicting the behaviour of cells in vivo. The method of studying attachment of cells to cryostat sections was first used to show a specific adhesion of lymphoid cells to post-capillary venular endothelium (Woodruff et al, 1977). The rationale for using this method is the assumption that the type of cell interaction involved in metastasis is likely to be mediated through the cell surface and that this may be detected by changes in cell adhesion. The preservation of cell surface molecules is readily apparent in many immunocytochemical applications. A problem with this method is the limited area of membrane presented by the sectioned cells. Apparent specificity of F10 cells for lung in this type of assay has already been reported (Netland & Zetter, 1984). In the present study there was no correlation between lung

adhesion and the number of lung tumours formed after IV injection. As the method followed was essentially similar it is difficult to account for this difference. It may have been expected that adhesion in this type of assay would have correlated more directly with local growth after direct injection. The F1 and F10 cells adhered equally well to the peritoneal cavity and the F1 P16 were less adherent. However F10 P18 which grew best at this site were also less adherent, not differing significantly from the F1 P16 line. The failure of this assay to be predictive of in vivo behaviour is also found with the polyoma salivary tumour. Adhesion of these cells to the peritoneal cavity where the cells did not grow was not significantly different from this adhesion to the tumour itself.

The results of these experiments were much more complex than had been anticipated. However two distinct mechanisms were seen as important in determining the growth of metastasising cells. The first mechanisms is specificity of trapping at a particular site. This is exemplified by the difference in the F1 and F10 cell lines. The second is the effect of particular sites on tumour cells. This is shown by the total inhibition of growth of the polyoma cell by the cells of the peritoneal cavity and by the different ways in which the peritoneal cavity modified the behaviour of the F1 and F10 lines of the B16 melanoma.

The next chapter in this thesis will consider the role which the histocompatability system could play in mediating this type of cell interaction.

CHAPTER THREE

The role of histocompatability antigens in mediating the cell interactions involved in the growth of metastatic tumour cells.

CHAPTER 3

3.1. INTRODUCTION

The study of the cell surface proteins collectively known as the histocompatibility antigens has its origin in the study of graft rejection. One group of genes was found to be related to rapid rejection and was termed the major histocompatability locus (MHC). However, it was soon realised that a large number of other genes could mediate less rapid rejection and these are known as the minor histocompatability loci. Although the mouse and human systems are best characterised there is evidence for polymorphic cell recognition systems in virtually all metazoan species. For example such a system prevents incompatible sponges being grafted (Hildemann et al, 1979). The mouse major histo-compatability complex (H-2) is found on chromosome 17 and the structure of this region is now known in detail (Steinmetz & Hood, 1983, Klein, 1979). The H-2 complex contains genes of three distinct classes; the class I genes K, D and L are located at either end of the complex, the class II genes are a group of four separate genes; A alpha, A beta, E alpha and E beta and finally the class III genes which code for complement components. Adjacent to the H-2 complex is the TLa complex which contains about 15 minor histocompatability genes as well as a number of non-functional pseudo-genes with class I homology (Winoto et al, 1983) (see Appendix 3). Functional and non-functional genes can be identified using transfection into L cells and measurements of class I expression by detection and quantitation of surface β2 microglobulin (β2 micro- globulin is the invariant component of all Class I genes).

Although there is a concentration of histocompatability genes on chromosome 17 class I genes are found on all mouse chromosomes.

The structure of the class I and class II genes and their protein products is well characterised. Class I genes have 8 exons which code for a leader sequence (1), three external domains (2-4), a transmembrane sequence (5) and a cytoplasmic domain (6-8) (Steinmetz & Hood, 1983 and (Steinmetz et al, 1981a). When incorporated into the membrane, class I molecules are associated with β2-microglobulin. There is some evidence that alternative mRNA splicing of some class I gene transcripts could produce different cytoplasmic domains (Steinmetz et al, 1981b). The class II molecules (I-A and I-E) consists of alpha and beta chains both of which bear some similarity to class I molecules. A similarity of Class II molecules to the T-cell receptor, immunoglobulin and Thy 1 and Lyt 2 lymphocyte differentiation molecules has been noted and it has been postulated that evolutionary relationships exist between these molecules (Hood et al, 1985).

The role of the H-2 locus in the mediation of cellular interactions has been studied in most detail in the immune system. This stems from the work of Zinkernagel & Doherty who found that cytotoxic T-cell activity against virus infected cells was only effective if both T cells and target cell shared the same H-2 class I antigens (Zinkernagel & Doherty, 1979). As is now well known this work was extended to show that antigen presentation by the mononuclear phagocyte system to T cells was restricted by the class II H-2 antigens. These observations are of

fundamental importantance in immunology but they have a wider significance in that they show that the function of histocompatibility antigens can be studied in at least some cases by the demonstration of strain specific restriction effects.

The detailed knowledge of histocompatibility antigen genetics and structure contrasts with the paucity of information as to how cells recognise these proteins. The identification of the T cell antigen receptor alpha, beta and gamma chains may have been expected to illuminate this question but as yet there is no consensus as to the role which the T cell antigen receptor plays in major histocompatibility locus recognition (Robertson, 1985). It is unclear whether recognition of H-2 proteins is mediated through the T-cell receptor or whether other surface molecules are required in particular the CD4 and CD8 differentiation antigen (Golding et al, 1985). Nothing is known about the recognition of non H-2 histocompatability antigens.

Cell interactions, other than those involving the immune system have been studied in little detail. When primary cultures of renal tubular epithelia derived from different strains of mouse are admixed it can be shown that contact inhibition of movement is strain restricted probably by either the K or D class I molecules (Curtis and Rooney, 1979). In this case contact inhibition was increased between allogeneic cells. The role of H-2 in modulating in vitro cell adhesion has also been studied (Bartlett & Edidin, 1978) using a collecting type assay. When cells of the same H-2 type were mixed variation in

degree of adhesiveness was found to be a function of H-2 polymorphisms. Secondly, adhesion was strongly inhibited by the presence of anti-H-2 antibodies. Thirdly, a significant difference was found between the adhesion of cells derived from hybrid animals to each other and adhesion to one of the parental types. Histocompatibility antigens were also found to be important in the adhesion of liver and bone marrow cells (Zeleny et al, 1978). In vitro studies of lymphocyte behaviour have shown that B-cell adhesion was inhibited by a thymocyte derived factor (Curtis & deSousa, 1973). Later it was found that when minute amounts of this factor were injected intravenously a rapid B-lymphocytosis occurred (Davies & Curtis, 1981). Purification of this factor by affinity chromotography showed that it was identical to part of the class I MHC molecule H-2D (Curtis & Davies, 1981). However, little is known about the conditions which lead to the release of this molecule in vivo.

These studies strongly suggest that histocompatibility antigens are involved in cellular processes other than the immune response. Indeed it has been demonstrated that H-2 molecules can be detected by odour and can affect mating preferences of mice (Beauchamp et al, 1985).

Studies of histocompatability antigen expression by tumour cells have tended to focus on the hypothesis of immune surveillance and tumour immunity rather than on the nature of cellular interactions. In this chapter the expression and function of histocompatability antigens will be examined in the B16 melanoma model with particular reference to metastatic potential and growth in distant

sites. The question will be approached in two ways. Firstly the highly metastatic lung homing B16 F10 cell line will be compared with the poorly metastatic non-lung specific B16 F1 cell line with respect to both H-2 class I and class II MHC expression. Secondly the effect ofincreased expression of these molecules, induced by retinoic acid, will be studied in vivo. Thirdly the ability of the highly metastatic F10 cell line to grow in animals of various genotypes will be studied in an effort to demonstrate strain-related restriction effects similar to those discussed above.

MATERIALS AND METHODS

The general methods of cell culture and the conduct of animal experiments were identical to those described in Chapter 2. The same methods were also used to assess lung and peritoneal metastasis and cell trapping by the lungs.

a) The effect of mouse genotype on metastasis formation.

To test the effects of varying the genotype of the recipient animal on the growth of injected cells the following experiments were performed. Two groups of animals were initially used. Group A consisted of C57 BL6 mice (the syngeneic strain for the Bl6 melanoma) and Group B consisted of Fl hybrids which resulted from the mating of C57 BL6 with balb c. These animals were each injected with 2 x 10^5 Bl6 Fl0 cells as described previously. The cells used were derived from the same culture flasks. The number of tumour deposits in the lungs of these animals was compared using Student's t-test. These experiments were then repeated using Fl hybrid animals which were produced by C57 BL6 x balb b mating (H-2b homozygous). See Appendix 4 .

Comparison of the number of peritoneal deposits resulting from the intraperitoneal injection of 2 x 10^5 F10 cells was also made using the C57 BL6 and Fl hybrid animals.

Syngeneic and C57 BL6 x balb c animals were compared with respect to the trapping of radioactively labelled Bl6 F10 cells in the lungs. The mean radioactivity in each group of animals was compared using Student's t-test.

b) Comparison of H-2 expression on Fl and F10 cells.

The expression of H-2 class I (K^bD^b) and I-Ab antigens

expression were assessed by immunofluorescent staining. Two mouse monoclonal antibodies were used for this purpose. Clone 5041 - 16.3 recognises a shared determinent on both the K and D products of the b haplotype. The MRC OX-3 monoclonal antibody recognises the I-A b haplotype product as well as the Ia product of some rat strains. Both antibodies were supplied as ascites fluid by Serotec Limited.

Four to six million B16 F10 or F1 cells were prepared as above, harvested, pooled and washed in MEM and then resuspended in 0.2 ml of calcium and magnesium free Dulbecco's phosphate buffered saline. This suspension was divided into two aliquots each of 0.1 ml and to one of these was added 15µl of undiluted antiserum. These suspensions were left on ice for 20 minutes. The cells were then pelleted by centrifugation and washed twice in Dulbecco's PBS containing 0.05% sodium azide and 0.1% bovine serum albumin. They were then resuspended in 0.1 ml PBS to which 20µl of rabbit anti-mouse immunoglobulin - FITC conjugate (Dako Ltd) was added. This was incubated on ice for a further 30 minutes and the cells were then washed three times in PBS containing 0.05% sodium azide and resuspended to about 106 cells per ml, in phosphate buffered saline. A small aliquot was placed on a glass slide and examined under ultraviolet light.

c) Quantification of Fluorescence Intensity.

In these experiments only cells stained and measured at the same time were compared.

The cell suspensions prepared as above were analysed using the fluorescence activated cell sorter (FACS II,

Becton Dickinson) which employs a 500mW Argon laser for maximum fluorescence activation and photomultiplier at 450V. About 10^5 cells from each sample were counted and measured. Cells which had not been treated with specific antibodies served as a measure of autofluorescence and non-specific binding of the rabbit anti-mouse - FITC. (Fig.21-22).

The FACS II recognises 256 channel steps in fluorescence intensity. The number of cells counted in each channel were used to calculate the number of cells at thirteen levels of intensity. This is analogous to varying the gain of the photomultiplier and making separate counts . The method of Fathman et al (1975) was used to subtract autofluorescence from each of these measurements so as to obtain a measure of the number of specifically fluorescent cells.

If E is the proportion of total cells detected at a particular level of fluorescence intensity then this will include α, the proportion which stain non-specifically at this level and to β the proportion of the total which stain specifically.However since autofluorescence and specific staining are independent then $\alpha\beta$ will be the proportion in cells of both categories:

$$\text{thus} \quad E = \alpha + \beta - \alpha\beta$$
$$\beta = \frac{E-\alpha}{1-\alpha}$$

E is measured from the specifically stained sample and from the autofluorescence control. Therefore, β the proportion of cell specifically stained and hence the absolute number can be calculated.

To compare F1 and F10 cells Chi squared tests were performed using a fourfold table. The total number of positively stained cells with each antibody were compared as well as those stained with high intensity defined as fluorescence intensity greater than that detected by Channel 121.

To examine the possibility that differences in fluorescent intensity were related to cell size, plots of intensity versus cell size were obtained using the FACS II system.

d) Induction of cells with retinoic acid.

The aim of these experiments was to examine the possibility that retinoic acid would induce MHC expression and so alter the in vivo metastatic potential of the cells.

F1 and F10 cells were plated as before. After 3 days retinoic acid was added and the cells used 5 days later. Retinoic acid stock solutions were prepared from crystalline retinoic acid (Sigma) dissolved in 95% ethanol: 5% water v/v.

The effects of retinoic acid on growth were assessed as follows. Replicate $25cm^2$ cultures were prepared and varying concentrations (10^{-6} to $10^{-10}M$) of retinoic acid added. After 5 days cells were removed and counted. The results were expressed as cells per $25cm^2$ flask for each concentration of retinoic acid and mean and standard error were calculated. These were compared with untreated controls using a t-test.

The expression of H-2 (K and D) and I-A were assessed as described above using cells which had been treated for 5

days with either 10^{-6}M or 10^{-9}M concentrations of retinoic acid. The results were then analysed following the methods described above. Again only cells stained and measured on the same day were compared.

To test the effects of retinoic acid on in vivo growth groups of C57 BL6 mice were injected intraperitoneally and intravenously with F10 and F1 cells induced with 10^{-6} and 10^{-9}M retinoic acid. The number of tumours were assessed as described above.

The trapping of cells in the lung was studied using radiolabelled cells induced with 10^{-6} and 10^{-9}M retinoic acid and compared with uninduced cells.

e) Assay for natural killer cell activity. (Appendix 5).

In these experiments B16 F10 cells were used as targets. These were radiolabelled as described previously with ^{51}Cr. 5 x 10^4 cells in 50µl of media were added to each well of a microtitre plate.

Effector cells were prepared from the spleens of C57 BL6 (N = 6) and C57 BL6 x balb c (N = 6) F1 hybrid animals. The spleens were removed and cut into three pieces. They were then gently squeezed to remove lymphoid cells which were suspended in Ham's F10 medium containing 10% foetal calf serum. These cells were then incubated at 37°C for one hour to remove plastic adherent cells. The cells remaining in suspension were then counted and resuspended.

Spleen cells prepared in this way were then added to wells containing the radiolabelled B16 melanoma cells. Each well received either 5 x 10^6 or 1.6 x 10^6 spleen cells in 150 µl of standard medium. As controls B16 cells were

incubated either with 150 µl of medium or with 150 µl of phosphate buffered saline containing 10% Nonindet P-40 (Sigma).

After two hours 100 µl of medium was removed from each microtitre well and counted in a gamma counter. The radio-activity from cells which had been treated with detergent was taken as the maximum possible release. The radioactivity from cells treated with medium alone was taken as the spontaneous release. These values were then used to calculate the cytotoxic effect of the spleen cells. The cytotoxicity of the syngeneic and F1 hybrid spleen cells was compared.

3.3. RESULTS

a) Expression of major histocompatability antigens by F1 and F10 cells.

1. Immunofluorescence Microscopy

It was apparent on ultraviolet microscopy that there was considerable heterogeneity of staining of tumour cells. Many cells were unstained by either antibody. Positive staining varied from a few tiny dots on the cell surface to near continuous membrane staining. This was similar with both antisera used. In control suspensions, where the primary antisera had been omitted, no bright green fluorescence was ever observed although a dull green autofluorescence was sometimes seen in the cell cytoplasm.

2. Fluorescence activated cell sorter analysis.

i. Staining with anti-H-2^b, K and D (clone 5041 - 16.3 monoclonal antibody). (Figs. 21 and 22).

The number of positively staining cells was calculated as described above. With this antibody 7535 of 8661 (87%) of F10 cells were positively stained after subtraction of autofluorescence. Cultures of F1 cells stained and measured under identical conditions showed positive staining in 4937 of 9831 cells (53%). Testing the null hyposthesis that there is no difference between these populations, Chi squared = 245.4; $p < 0.0001$.

When only brightly stained cells are considered (intensity greater than channel 121) 2685 from 8611 (31%) of F10 cells were positive as compared to 1735 of 7596 (23%) of F1 cells. Chi squared in this case is 373.12 $p < 0.0001$. Thus there is a highly significant difference in the number of cells of each type staining with this antibody.

ii. Staining with anti-I-A^b (monoclonal antibody MRC-OX3).

Using this antibody 6489 of 9271 (67%) of F10 cells showed positive staining as opposed to 4350 of 9802 (44%) of F1 cells measured. Chi squared in this case was 281.25; $p < 0.001$. When high intensity staining alone was considered 649 of 9271 (7%) of F10 cells were stained compared with 252 of 9550 (3%) of F1 cells, Chi squared = 201 p $0 < .001$.

These results show that significantly more F10 cells express H-2 KD and I-A than F1 cells. However it is noticable that many fewer cells express the I-A determinant than the class I proteins and that very few cells expressed I-A with high intensity.

This experiment has been repeated subsequently on several occasions with qualitatively similar results. There was no apparent relationship between cell size and fluorescence intensity (Fig. 23-25).

b) Induction of B16 cells with retinoic acid.

i. Cell growth.

Cells incubated for 5 days in concentrations of retinoic acid from 10^{-6} to 10^{-10}M show variable dose-related inhibition of growth. At 10^{-6}M the final cell density was almost one third of the control value. Using a t-test this was significant at $p<0.01$. Cells grown at 10^{-7} and 10^{-8}M retinoic acid also showed significant inhibition of growth $p < 0.05$. There was no significant inhibition at 10^{-9} or 10^{-10}M retinoic acid (Fig. 26).

ii. Cell morphology.

No apparent difference in the appearance of either F1 or F10 cells could be detected after treatment with

retinoic acid. A rather inconstant finding was the presence of excess melanin pigment in the medium; most cultures treated with 10^{-6}M retinoic acid showing marked discoloration after 3 - 4 days.

c) Effect of retinoic acid on expression of H-2 KD and I-A determinants.

i. Cells treated with 10^{-6}M retinoic acid.

After induction with 10^{-6}M retinoic acid an increase of 38% in the number of F1 cells staining with the anti-H-2 K,D antiserum was found. 18% more cells showed high intensity fluorescence (Fig. 27). Using the anti-I-A antibody 22% more retinoic acid treated cells were stained that the uninduced controls (Fig. 28). A 3% increase was found at high intensity (Fig. 28).

In each case the difference between induced and non-induced cells is significant ($p<0.001$) using Chi squared test.

A similar pattern was seen with F10 cells treated with this concentration of retinoic acid. An 8% increase in the number of cells stained with the antibody to class I determinants and a 4% increase in cells staining at high intenity was found (Fig. 29). Using the anti-I-A antibody 8% more induced cells were stained than uninduced cells. At high intensity the increase was 2% (Fig. 30).

The increase in the number of cells stained with each antibody after induction was significant ($p<0.0001$) using Chi squared test.

ii. Cells treated with 10^{-9}M Retinoic Acid.

Using retinoic acid at 10^{-9}M there was no change in the number of F1 cells staining with anti-H-2K,D. A 3%

decrease in the number of cells stained with anti-T-A was found. This was not statistically significant (Fig. 31).

Induction of F10 cells with 10^{-9}M retinoic acid produced a 3% increase in staining with anti-H-2 (KD) which was not significant (Fig. 32). However the number of cells stained with anti-I-A increased by 18% which was significant at $p < 0.001$ using Chi squared test.

d) The effect of retinoic acid induction on the tumour forming capacity of F10 and F1 cells.

i. Intravenous Injection.

B16 and F1 cells were induced for 5 days as described above with 10^{-6}M and 10^{-9}M retinoic acid. Following intravenous injection of both F1 and F10 cells induced with 10^{-6}M retinoic acid no tumour deposits were found in the lungs of 10 animals injected with each cell line. With F10 cells induced with 10^{-9}M retinoic acid a mean of 16.7 ± 5.8 ($n = 10$) tumours per animal were found (Fig. 33). When compared with uninduced F10 cells this was a significant decrease (Student's t-test $p < 0.0001$). When this experiment was repeated using F1 cells at 10^{-9}M retinoic acid a mean of 1.5 ± 0.5 ($n = 10$) tumours per animal was found. This was not significantly different from the uninduced F1 cells Fig. 34).

The lungs of animals showing no tumour were carefully examined histologically to exclude the possibility that failure to observe tumour on the pleural surface was due to a reduced rate of growth. However, there was no evidence of tumour in any of these animals.

ii. Intraperitoneal injection.

Retinoic acid treated F10 and F1 cells were used in

the same protocol as described above. Again when 10^{-6}M retinoic acid was used no tumours were seen in the peritoneal cavity with either cell line. When F10 cells were induced with 10^{-9}M retinoic acid a mean of 21 ± 4.5 (n = 10) tumours per animal was found. This is a significant reduction compared to the uninduced F10 cells at $P<0.001$ (Fig.35). When 10^{-9}M retinoic acid F1 cells were used a significant decrease in the number of tumours (44.9 ± 8.5 (n = 10)) was also observed $P<0.05$ (Fig. 36).

iii. The effect of retinoic acid induction in the trapping of B16 F1 and F10 in the lungs.

Cells which had been induced with retinoic acid were used in the same assay as previously described. B 16 F10 cells induced with 10^{-6}M retinoic acid gave a mean lung radioactivity of 1149 ± 107 cpm/g (N =12) at 2 hours. This was a significant reduction ($p < 0.001$) as compared to untreated cells at 2 hours (3350cpm/g). F1 cells induced by 10^{-6}M retinoic acid showed a marked rise in lung radioactivity with a mean of 3984 ± 590 (N = 10). This was significantly greater ($p < 0.001$) than in the control animals (Figs.37-38).

Labelled B16 F1 cells treated with 10^{-9}M retinoic acid gives a mean lung radioactivity of 1413 ± 248 (cpm/g) (10 = N). This is not significantly different from the uninduced F1 cells. When labelled F10 cells induced with 10^{-9}M retinoic acid were used a mean lung radioactivity of 2478 ± 416 cpm/g was found. This was not significantly different from the uninduced F10 cells (Figs. 37,38).

In summary F1 cells induced with 10^{-6}M retinoic acid showed greatly increased trapping. The levels were

comparable to that seen with uninduced F10 cells. However induction of F10 cells at this concentration resulted in a decrease in lung trapping. At 10^{-9}M retinoic acid both cell lines showed similar levels of lung trapping as the uninduced cells.

e) The effect of recipient genotype on the metastatic potential of F10 cells.

i. Intravenous injection.

Injection of 2×10^5 B16 F10 cells intravenously give a mean number of lung colonies of 212 ± 23 in the syngeneic C57 BL6 mouse. In the C57 BL6 x balb c hybrid animal the corresponding number of lesions was 20.5 ± 4 (N = 21). This is significantly less than in the syngeneic animals at ($p < 0.0001$). The experiment was repeated using C57 BL6 x balb b ($H\text{-}2^b$ homozygous) hybrids and the mean number of lesions found was 23.2 ± 7.2. This did not differ significantly from the C57 BL6 x balb c hybrids. Tumours were not seen in any other organ other than the lung (Fig. 39).

ii) Intraperitoneal injection.

The number of tumour deposits in the small intestinal mesentery was compared in the C57 BL6 and C57 BL6 x balb c hybrids. The C57 BL6 showed a mean number of tumours of 66.5 ± 5.1 (N = 15) and the hybrid animals a mean of 63 ± 5.8 (N = 15). This difference was not significant (Fig. 40).

iii) Trapping of radiolabelled F10 cells in syngeneic and F1 hybrid animals.

Radiolabelled F10 cells were injected intravenously into C57 BL6 x balb c hybrids and the retained lung radio-

activity compared at two hours and four hours. The mean lung radioactivity at two hours in the hybrid animals was 4090 ± 341 cpm/g (N = 18) as compared to 3356 ± 352 cpm/g (N = 18) in the syngeneic animals. Although the mean obtained in the hybrid animals was rather higher the difference was not significant (Fig. 41).

At 4 hours the results were similar. The C57 B16 x balb c hybrid had a lung radioactivity of 616 ± 64 (N = 19) as compared to the C57 BL6 control animals which showed a mean of 463 ± 74 cpm/g. This again is not a statistically significant difference (Fig. 42).

f) Detection of anti-B16 F10 natural killer cell activity.

Using B16 F10 cells as targets, spleen cells from C57 BL6 and C57 BL6 x balb c hybrids were tested for natural killer cell activity. In neither case was there any evidence of significant NK cell mediated lysis of the B16 cells.

DISCUSSION

It is apparent from the immunofluorescence data that a significantly greater proportion of B16 F10 cells stained positively with both the anti-KD and the anti-I-A antibodies than the B16 F1 cells. The antibodies used in this study were obained from a commercial source. In both cases the antigenic specificity of the monoclonal antibodies had been determined by cytotoxicity analysis of cells from recombinant mouse strains (manfacturers data). MRC-OX3 has also been used in immunoprecipitation studies of I-A antigens (McMaster and Williams, 1979). As the characterisation of these antibodies was carried out using spleen cells, it remains a possibility that they could recognise a cross-reacting determinant on the B16 melanoma cells. It was hoped to exclude this by performing "Western" blot analysis of separated B16 proteins. However, unfortunately neither antibody performed well in this analysis, even at high concentrations. Therefore, in the following discussion the not unreasonable assumption is made that the antibody specificity in B16 melanoma is the same as has been described in spleen cells. The data illustrated in figures 21-32 are representative of a series of experiments conducted using the B16 cell lines stained with these antibodies. As these results were obtained at different times it is not possible to pool the data. However in each case the results were qualitatively similar.

The expression of class I MHC molecules on tumours is relatively common, however class II (Ia) expression has seldom been reported. Class II expression in normal cells

is now thought to be more widespread that has been generally realised (Natali et al, 1985). Consistent with the results of this study is the finding that class II molecules are relatively frequently seen in human malignant melanomas although not in epidermal melanocytes (Wilson et al, 1979, Natali et al, 1985, Burchiel et al, 1982). In human cultured cell lines HLA-DR expression in neoplastic melanocytes correlates with the ability to stimulate autologous lymphocytes in vitro (Guerry et al, 1984). However, the relevance of such studies to in vivo tumour behaviour must be questioned. Immunocytochemical studies have also shown HLA-DR to be present on poorly differentiated colon carcinomas (Rogum et al, 1983) and in a minority of gynaecological tumours (Ferguson et al, 1985). In the latter study there was no obvious relationship between HLA-DR expression and lymphocyte infiltration. It has been postulated that non-lymphoid cells which aberrantly express class II molecules are also able to present surface proteins to T cells and thus evoke an immune reponse (Botazzo et al, 1983). This hypothesis does not satisfactorily explain why cells which normally express class II MHC do not behave in this way. Furthermore, attempts to test this hypothesis have not satisfactorily excluded the possibility that HLA-DR expression may be related to cell regeneration following injury. In the case of B16 F10 cells, which in vitro expressed relatively high levels of class II MHC, there was no histological evidence of a significant immune response to solid tumour deposits, although it did not prove possible to demonstrate expression in vivo by

immunocytochemistry. Therefore, at the present time the functional significance of class II MHC in B16 melanoma and other tumours remains unclear.

Immunocytochemical studies of human tumours have not revealed a consistent alteration in the expression of class I molecules in malignancy or in the degree of inflammatory response to the tumour. However, unlike class II molecules, a number of experimental mouse tumours have been studied which provide a basis for discussion of the results of this experiment. Taken as a whole, aggressive and metastatic mouse tumour cell lines tend to have higher levels of class I MHC expression. This is consistent with the data presented here which show that the highly metastatic and lung homing F10 cell line had significantly increased expression of H-2 K and D than the similar, but poorly metastatic, F1 cell line. The detailed study of a number of other cell lines has led to the description of several mechanisms by which changes in H-2 expression could lead to alterations in tumour behaviour. One system which gives different results from the B16 melanoma are the tumours produced by adenovirus infection. When rat cells are infected with either Ad12 or Ad5 which are similar viruses only the former is able to produce tumours when injected into animals (Schrier et al, 1983). This is correlated with absence of class I MHC molecules from the cell surface. The same experiment can be performed with murine cells and it can be shown that Ad12 infected cells are tumorogenic even across allogenic boundaries. However, the oncogenic property of Ad12 infected cells is abrogated when the H-2 L gene (Class I) is transfected with a promoter and enhancer

(Tanaka et al, 1985). The explanation for this phenomenon is that cytotoxic T cells do not recognise the Ad12 antigen on the cell surface in the absence of H-2 class I molecules and so the cells are protected from immune destruction (Bernards et al, 1983).

A tumour cell line in which the more aggressive variant has higher H-2 expression is the Eb/ESb T cell lymphoma. This cell line originated from a chemically induced tumour (Schirrmacher et al, 1984). The highly metastatic ESb line expressed both H-2 K and D at a higher level than the less aggressive Eb line (Schirrmacher et al, 1984). There is some evidence that the ESb line has a higher degree glycosylation of H-2 than Eb. As a methylcholanthrene induced tumour Eb/ESb expresses tumour associated transplantation antigens which are different for the two cell lines, but both evoke a T cell response. Thus clearly the ESb cells have other mechanisms of immune escape than failure to express class I MHC. Although B16 melanoma cells are not known to express tumour associated transplantation antigens, in the correlation of metastasis with H-2 class I expression they appear to resemble the Eb/ESb system.

A further pattern of H-2 expression is seen in a number of cell lines which show an altered ratio of H-2 D to H-2 K. This phenomenon is seen in the Lewis lung carcinoma (3LL), a spontaneously arising tumour of C57 BL6. When a metastatic and a non-metastatic cell lines were compared it was found that no H-2 K was present on the metastatic cell line, but that H-2 D was present in both (Eisenbach et al, 1983). This work was extended by

producing multiple clones from this tumour and examining H-2 expression and metastatic potential. A relatively good correlation was found between the D:K ratio and the ability to form metastasis (Eisenbach et al, 1984).

This type of relationship has been found in a number of other mouse tumours. The T10 sarcoma metastatic variants show higher D than K expression (Wallich et al, 1985). Furthermore correction of this D:K imbalance by transfecting K genes into T-10 cells resulted in cells which were unable to metastasise (Wallich et al, 1985). The proposed mechanism by which this imbalance effects cell behaviour is through the abolition of the immune response. The evidence cited to support this is twofold. First in the case of the T10 sarcoma cells expressing H-2 K cells were able to metastasise in mice which has been irradiated (Wallich et al, 1985). Some caution is necessary in interpreting this as evidence of an immunological mechanism. It has been shown that local irradiation without demonstrable effects on immunity can lead to much higher metastasis of tumour to that particular area (Milas & Peters, 1984) the mechanism perhaps being mediated through local vascular injury. The second line of evidence are the observations made in AKR leukaemia cell lines. The K36 line which is H-2K deficient and rapidly growing is resistant to T cell mediated cytotoxicity. Transfection of the K gene restores sensitivity to T cell lysis (Hui et al, 1984). This suggests that K is much more effective than D in presenting viral associated antigens to cytotoxic T cells (especially in the case of Gross leukaemia virus associated to tumours). Low levels of H-2K and resistance

to in vitro lysis are a feature of many other AKR leukaemias, but several lines exist which express K and are lysed in vitro, but grow aggressively in vivo (Schmidt & Festenstein, 1982) (Plata, 1982). The AKR cell lines are also very unusual in that some may show expression of a new H-2 haplotype. The mechanism of this is not understood (Festenstein & Schmidt, 1981).

The antibody which is used in this study to measure H-2 class I expression recognised both K and D proteins and therefore the measured fluorescence was the sum of expression of these two molecules. Antibodies which would recognise K^b and D^b separately were not available. The B16 melanoma differs from the T10 sarcoma, AKR leukaemia and Eb lymphoma in that it was a spontaneously arising tumour and no tumour associated transplantation antigens have been described, nor has immune rejection been found to explain the differences in behaviour between the F10 and F1 cell lines. This raises the possibility that the elevated class I antigens may potentiate metastasis independent of the immune system. In the absence of specific antisera it was decided to attempt to alter the expression of H-2D by treatment of B16 cells with retinoic acid and observing changes in in vivo behaviour. The specific effect of retinoic acid in inducing elevated expression of H-2D has already been described in 3LL cells (Eisenbach et al, 1983).

When a concentration of retinoic acid identical to that used in the 3LL cell line (10^{-6}M retinoic acid) was added to the culture medium a significant increase in both class I and class II MHC proteins were observed in both the

F10 and F1 cell lines. Increase in MHC class II has not previously been described as an effect of retinoic acid. Although significantly greater numbers of F1 and F10 cells express these proteins, the magnitude of increase was much greater in the case of the F1 cell line. When the effects of retinoic acid were examined *in vivo* and *in vitro* the results were quite different from those seen with the 3LL cell line. At 10^{-6}M retinoic acid cell growth was greatly inhibited and the formation of tumours following intravenous or intraperitoneal injection was reduced almost to zero. Careful histological examination of tissue from these animals showed no evidence of microscopic deposits of tumour which may have been expected if the effect of retinoic acid was solely to inhibit the rate of growth. As a result of these studies a titration of retinoic acid against *in vitro* growth was performed. This showed that growth inhibition ceased to be significant at 10^{-9}M retinoic acid. When the immunofluorescence studies were repeated at this concentration it was found that there was no significant increase in MHC class I in either cell line. However even at this concentration there was a marked decrease in the number of tumours formed following intravenous or intraperitoneal injection. This experiment shows that in the B16 melanoma system retinoic acid inhibits metastasis formation by a mechanism which is independent of its inhibition of growth *in vitro* and of its induction effect on MHC class I and class II expression. It has been suggested that retinoic acid may effect tumour progression in metastasis behaviour *in vivo* by virtue of is presumed immunostimulatory effects (Eccles, 1983). This was

clearly not the case here as retinoic acid was not injected into the animals. Thus it appears that retinoic acid is able to induce irreversible or at least long lived inhibition of tumorogenicity in these cells.

From these studies it is apparent that retinoic acid has multiple effects on this cell line which makes it impossible to disentangle the effects of increased expression of MHC class I and class II and in vivo tumour formation. However, one variable which is independent of growth potential and appear to correlate well with metastasis formation is trapping of cells in the lungs following intravenous injection. Uninduced F10 cells were trapped more avidly than F1 cells in keeping with their differing metastatic potentials (Chapter 2). When the trapping of retinoic acid induced cells was studied it was found that trapping of F1 cells was greatly increased at 10^{-6}M retinoic acid but that 10^{-9}M retinoic acid had no significant effect. This correlates with the changes in MHC expression seen at these concentrations of retinoic acid. However, the opposite was observed with F10 cells which showed a small inhibition of cell trapping at 10^{-6}M retinoic acid again returning to control levels at 10^{-9}M retinoic acid. One possible explanation for these results could be that the large increase in MHC seen in the F1 cells following treatment with 10^{-6}M retinoic acid was sufficient to overcome an opposing effect and so increase cell trapping. In the F10 cells only a small stimulation of MHC was produced and so net inhibition of trapping could have resulted. However, in view of the complexity of the effects of retinoic acid in this system a number of other explanations are equally possible.

An alternative method of studying the involvement of histocompatability molecules in the process of tumour metastasis is to attempt to show whether metastatic growth is restricted when animals of different genotypes are used. This is analogous to methods used to study in vitro cell interactions (Zinkernagel and Doherty, 1979). The initial approach to this question was to study the number of tumours produced when B16 F10 cells were injected into a semi-allogeneic animal. The animal chosen was a hybrid of C57 BL6 and balb c. (Appendix 4). This animal is heterozygous at all known polymorphic histocompatability loci but would accept tumours without immunological rejection (the tumours in this case being equivalent to a graft from one of the parental animals). It was found that the number of tumours produced in the lungs of these animals following IV injection was very much less than in the syngeneic recipients. In the hybrid animals there was no evidence of tumour deposits outwith the lungs and in this respect tumour cells behaved as they did in the syngeneic animal. These results would suggest that a strain related restriction effect was indeed present but that it did not effect the preference of the tumour cells for growth at a particular site.

To attempt to further define the locus involved in this restriction effect hybrids were prepared by mating balb b with C57 BL6. The balb b strain is congeneic with the C57 BL6 at the H-2 complex but in other respects is identical to the balb c mouse. Thus, these hybrid animals were homozygous at the H-2 locus only. When B16 F10 cells were injected into these animals a large reduction in the

number of metastasis was also observed similar to that seen with the C57 BL6 x balb c hybrid. This suggested that a restriction effect was present due to a locus other than H-2. It does not exclude the possibility that the H-2 locus was also involved, merely that it was not solely responsible for the restriction effect. In order to test whether an H-2 mediated restriction effect was also present, it would have been necessary to test hybrids which were heterogenous at H-2 but were homozygous at all other loci. C57 Bl6 derived strains congenic at H-2 are not available, and unfortunately the closely related C57 Bl 10 congenic strain are known to differ at a number of loci. (Appendix 4).

Although it is not likely that an immune response could account for the difference observed between the syngeneic and hybrid animals it was possible that natural killer cell activity may have been responsible. Strain differences in NK activity are known to occur (Hanna and Fidler, 1981). To exclude this possibility spleen cells from C57 BL6 and hybrid animals were tested against F10 targets but no activity was found. Variable degree of resistance of F10 cells to NK lysis has previously been reported (Hanna and Fidler, 1981, Erlich et al, 1984, Talmadge et al, 1980).

To characterise the restriction effect further, cells were injected directly into peritoneal cavity of C57 BL6 x balb c hybrids. In contrast to the results of intravenous injection there was no difference between the hybrid animals and the syngeneic recipients. When ^{51}Cr radio-labelled cells were injected a similar degree of lung

trapping was observed at 2 hours and 4 hours in both types of animal. These results raise the question as to the stage of the metastatic process at which the restriction effect is operative. After being trapped in the pulmonary capillaries the tumour cells must emigrate into the lung parenchyma, attach and begin to grow. If the factors which allow growth in the the peritoneal cavity are the same as those in the lung, then the stage of emigration from vessels would seem to be the likely point at which the restriction effect is acting. In view of the discussion in chapters 2 and 4 of local tissue effects on tumour growth, this assumption may not be justified and it remains possible that a factor involved in lung growth specificity may be responsible for the restriction effect. The idea is difficult to test as there is no comparable method of testing the effects of lung tissue on growth, direct injection being impractical.

In view of these results it is pertinent to ask whether this phenomenon has been observed in other types of metastasising tumour. The T10 sarcoma is a heterozygous tumour ($H\text{-}2^{d} \times H\text{-}2^{k}$). It has been shown that in syngeneic i.e. hybrid animals metastasis will occur only if the tumour cells express both parental haplotypes (Katsav et al, 1981) (DeBaetselier et al 1980). Clones were isolated from this tumour which expressed only one haplotype and were able to grow subcutaneously but did not metastasise (Katsav et al, 1983). Occasionally these clones spontaneously express both haplotypes and become metastatic. These effects were separate from the K:D imbalance described above. The authors of these studies

suggested that the results could be explained by an immunological mechanism. However, the only evidence for this was enhancement of metastasis by irradiation and, as has been discussed above, irradiation can potentiate metastasis without an effect on the immune system. Furthermore, it is difficult to reconcile the observed local growth of the non-metastatic clones with an immune mechanism. An alternative explanation is that the phenomenon seen in theT10 sarcoma is similar to the results described above in B16 melanoma in so far as both tumour and recipient organs lacked concordance of one allele. In both cases local growth was not impaired.

The Lewis lung carcinoma has been described in detail above. However, it is worthwhile considering the growth of this tumour in various strains. Unfortunately most studies using this tumour have assayed metastatic growth either by lung weight or uptake of ^{125}I-iododeoxyuridine rather than by direct counting of the number of metastasis. Thus, the end point of these studies is not identical to those described here. It was found that the 3LL tumour grew locally in syngeneic and allogeneic mice (Isakov et al, 1981) but that growth was best in syngeneic or semi-allogeneic recipients. Using congenic B10 strains differing at H-2 it was found that most of this effect was non-H-2 mediated. Spontaneous metastasis occurred in syngeneic and semi-allogeneic but not in allogeneic mice. Furthermore it was found that this appeared to be controlled by loci outwith the H-2 complex as well as by the H-2D locus. One criticism of these studies is that hybrid C57 B10 congenic strains were used although the

tumour is syngeneic with C57 BL6. As described above, in studying non-H-2 effects this difference may be significant.

Further experiments were performed to investigate the role of the immune system in these phenomena (Isakov et al, 1982). If neonatally tolerised C3H mice (which are allogeneic with 3LL) were injected with 3LL cells metastasis were found in about half the animals as compared to all the syngeneic animals (Isakov et al, 1982). (It should be noted that these results refer to spontaneous metastasis from footpad injection which is of course different from the method used in this study). Earlier work with this model made use of hybrid animals (Isakov et al, 1981) but these immune response experiments did not. Therefore, it is difficult to be certain that the same mechanism is operative. Furthermore, where an immune response is suspected lung weight and labelled Iododeoxyuridine uptake may not be the optimum measure of metastasis as inflammatory cell infiltration would produce changes in both these variables. However the results obtained with 3LL in hybrids are in some respects similar to those described in the Bl6 melanoma in this study. A striking point of similarity is that histocompatability antigens seem to be much less important to local growth than to metastasis.

Another model which should be considered is the Eb lymphoma; this again has been described above. Like the 3LL carcinoma interpretation of this work is very complex. In most of these experiments the end point is taken at time of death and it is stated that the animals were rarely

examined at post mortem (Schirrmacher, 1979). This is a less than satisfactory measure of metastasis but a number of points are worthy of note. It is stated that injection of one ESb cell into the syngeneic recipient (DBA/2) will be fatal in three weeks. The time taken for half the animals to die was $12\frac{1}{2}$ days after subcutaneous injection of 10^5 cells. When an F1 DBA/2 x B10 D2 was used the time taken for half the animals to die was 30 days. This hybrid is homozygous at H-2 but differs in the minor histocompatability loci (Schirrmacher, 1979). This tumour is chemically induced and expresses a tumour associated transplantation antigen. It was demonstrated that additional resistance to the tumour could be transferred by sensitised lymphocytes. The difference between syngeneic and hybrid animals was not further discussed in this study. The ESb tumour commonly metastasises to the liver (Schirrmacher et al, 1980) and in vitro forms rosettes with hepatocytes. This interaction can be blocked anti-H-2 sera. The results of this study again appear similar to those described here.

The experiments described in this chapter indicate that the products of both the H-2 locus and other strain related polymorphic loci may be involved in mediating the process of metastasis. These findings are consistent with the results of a number of other studies, although in some cases it is difficult to separate the effects of the immune response. Given the large number of H-2 congenic and recombinant mouse strains which are now available it should be possible to explore further the role of the MHC loci, although for the reasons given the B16 melanoma is probably

not the best model. For example it would be of interest to examine the question of K:D imbalance by varying the genotype of the animal rather than the tumour cells. This could be achieved using H-2 recombinant strains.Investigation of non-H-2 polymorphic loci is a much more difficult problem. These include but may not be entirely synonymous with the non-H-2 class I genes. The production of antisera to the gene products and useful mouse strains would be an enormous task, although if the products of these genes are important mediators of cell interactions it may prove to be rewarding.

CHAPTER FOUR

GENERAL DISCUSSION.

CHAPTER 4

GENERAL DISCUSSION

It is readily apparent from the preceding chapters of this thesis that the problem of tumour cell growth at different anatomical sites remains far from clearly understood. There is considerable debate on the general applicability of the various model systems which have been used to study this problem. Despite the obvious limitations of animal and cell culture models they remain useful as a source of ideas which can perhaps be tested in other ways. The experimental work described in chapters 2 and 3 approach the problem of tumour growth at distant sites from two distinct viewpoints. In chapter 2 the effect of particular environments on tumour cell behaviour was considered. In chapter 3 the role that one group of cell surface molecules may have in the mediation of these interactions was examined. From this work a number of ideas have emerged.

a) Cell trapping by the vasculature as a determinant of the site of tumour growth.

Previous studies have suggested that the difference in lung homing behaviour of the B16 F1 and F10 melanoma cells could be due to differences in the trapping of these cells in the pulmonary capillary bed (Fidler, 1975). A difference in the trapping of F1 and F10 cels in the lung was confirmed in chapter 2. However a number of pieces of evidence from the experimental studies in this thesis suggest that trapping by capillary endothelium cannot be the sole explanation for the differing behaviour of these cell lines. Firstly, there is an approximately threefold

difference in the trapping of F1 and F10 cells, but the number of tumours which arise in the lung after injection of F10 is about twenty times greater than after an injection of an equal number of F1 cells. A poor correlation of cell trapping with tumour formation was also seen in studies of B16 growth in transplanted lung and other tissues (Hart & Fidler, 1980). This discrepancy is further supported by two other observations. The cell line derived from the peritoneal passaged F1 cells (F1 P16) had a much greater lung colonising ability than its parent cell line, but without any difference in the level of trapping in the lung. Secondly, the F10 cells injected into C57 BL6 x balb c hybrid animals produced far fewer tumours but again with no change in the level of trapping. These studies suggest that while initial trapping may be important it cannot be the only determinant of the site at which a tumour will grow. In these experiments it is difficult to disentangle the relative contribution of initial vascular trapping from other factors which determine tumour cell growth. One model which would be useful in studying vascular trapping is the malignant lymphomas. Lymphocytes are naturally circulating cells (for review see deSousa, 1981). It has been suggested that trapping of lymphocytes by the high endothelial venules in lymphoid organs is central to the functioning of this circulation (Schoefl, 1972) (Woodruff et al, 1977). Indeed it is interesting in view of the results of Chapter 3 that the process of cell trapping by these vessels is MHC restricted (Degos et al, 1979). Lymphoid tumours, as large monoclonal populations, offer the opportunity to study

vascular trapping at particular sites. Some recent studies with human extranodal lymphomas indicate that this may be a useful approach. It has been found that lymphomas arising in thyroid, stomach and salivary gland remain localised for long periods before becoming disseminated. (Anscombe & Wright, 1985) (Compagno & Outel, 1980, Moore & Wright, 1984, Hyman & Wolfe, 1976). The demonstration of circulating cells in these tumours suggest that the neoplastic cells recirculate to their site of origin. The practical application of these studies is seen in the efficacy of surgical treatment of these tumours (Fleming et al, 1982).

b) The effect of tissue environment on tumour cell growth

The discrepancy between cell trapping and actual tumours arising in the lung points to the effect of the tissue environment in determining tumour cell growth. This finding can be further illustrated by the results of two further experiments described in chapter 2. Firstly, when cells from the benign salivary gland tumour were injected into the peritoneal cavity no evidence of tumour growth was found. Thus is can be concluded that the environment of the peritoneal cavity was not suitable for growth of these cells even although the tumour grew rapidly at its primary site. When small fragments of tumour were transplanted to this site these sometimes attached and the tumour cell survived but with little evidence of growth. This again suggests that the peritoneum was a non-permissive environment for the growth of this tumour although the tumour cells in association with their own stromal tissue were able to survive. A tumour which displays this type

of site restriction is obviously a potentially useful model for studying the mechanisms underlying this type of behaviour. It is unfortunate that the practical difficulties of the mouse salivary tumour model prevented this i.e. failure to culture the cells and lack of an inbred host.

Secondly, the repeated passage of F1 cells through the peritoneal cavity lead to the derivation of a cell line with quite different characteristics from its parent line (increased lung lesions, decreased peritoneal growth and poor in vitro proliferation). The change produced in this cell line was clearly of a more permanent nature than would be expected if the inhibited growth in the peritoneum was due, for example, to absence of a growth factor. Although repeated passage of a cultured cell line is clearly a highly artificial situation which is probably not comparable to any natural event it is of interest to consider what relevance these results may have for the phenomenon of tumour dormancy. Some of the clinical and experimental aspects of this condition are described in Chapter 1. It is possible that a particular tissue could cause profound suppression of previously rapidly growing tumour. This could result paradoxically in the emergence of a cell population with different properties perhaps by a mechanism analogous to the clonal instability phenomena described in B16 melanoma and other tumours (Poste et al, 1981). This is one explanation for the emergence and behaviour of the B16 F1 P16 cells as previously discussed.

c) Selective and inductive mechanisms

The original derivation of the B16 F10 cell line

involved repeated passage of cells which had formed pulmonary metastases following intravenous injection. In the experiments described in Chapter 2 cell lines of different properties to the F1 and F10 parent cell lines were derived by serial intraperitoneal passage. This raises the question as to whether these cells arose by selection of preformed sub-populations or by an inductive effect with peritoneal cavity. An inductive mechanism is one in which one tissue acts on another to alter its differentiation. It has two important elements firstly a signal and secondly a tissue competent to respond to this stimulus (for review see Slack, 1983). Such mechanisms are known to occur in embryogenesis.

There is some evidence to suggest a selective mechanism may be responsible at least for some of these observations. The study cited previously (Nicolson and Custed, 1982) where cells injected in microspheres were allowed to grow in the lung clearly favour the selective hypothesis. Repeated passage of the F10 cells resulted in a population which has a greater mesenteric tumour-forming ability but retained its lung colonising properties after intravenous injection. However after intravenous injection no tumours occurred in the peritoneal cavity. In this experiment growth at the site did not confer homing properties unless the vasculature is involved. As the interaction with vessels is a transient step this probably again favours a selective mechanism.

The behaviour of the F1 cells is less easily explained on a selective basis. It is not easy to conceive of selective pressures which could produce a stepwise decrease

in mesenteric tumour formation as well as severely impaired in vitro growth, the latter being a permanent change. An inductive mechanism would seem more likely to account for this type of behaviour. If this were true then competence to respond to the inducing signal would be an important difference between the F10 and F1 cell lines. This may also explain some of the discrepancy between trapping and tumour formation as described above if such an inductive mechanism also operated in the lung.

The long lasting changes in the F1 cell line following intraperitoneal passage may reflect genomic changes, for examples, methylation or even gene rearrangement. This could perhaps be studied using restriction enzyme polymorphisms although it would be a very laborious process. Such studies could however, provide an interesting insight into the mechanisms of actions of inducers with implications beyond the study of tumour biology.

Although these experiments are useful in so far as they raise the question of selection versus induction, it must be concluded that they do not go far toward separating the two effects.

d) The molecular basis of tumour cell growth at distant sites

Investigation of the molecular basis of tumour metastasis has proved to be difficult probably because the concentrations of the important proteins and messenger RNAs are below the detection level of currently available techniques. The approach adopted in Chapter 3 was to look for strain-related restriction effects similar to those

seen in the immune system. Evidence cited in the introduction to Chapter 3 suggested that such restrictions were not exclusively confined to the immune system but may be a property of interactions between many other types of cells. A strain-related restriction of F10 lung metastasis formation was indeed found but this was not due to MHC differences. Further careful review of other published studies suggested a similar effect has been observed but interestingly largely ignored in other tumour cell models. Without precise molecular identification it is impossible to be certain of the basis of this restriction effect. However one or more of the non-H-2 class I histocompatibility molecules would seem to be by far the most likely candidate. As yet class I and class II histocompatibility molecules are the only known mediators of restriction effects.

The possible involvement of a non-H-2 histocompatibility model is of particular interest in view of the great diversity of these molecules, at least 50 genes being known in the mouse. The possible role of histocompatibility molecules in relation to normal development has been discussed by Ohno (1977). Nothing is known about the control of these genes but it is obvious that a vast number of different combinations of their expression are possible and that this could be the basis of a complex system of cell interactions. The elucidation of this hypothesis depends on the availability of appropriate reagents and animal strains of a suitable genotype.

This thesis began by describing the clinical problems which had inspired the experimental study of metastasis. It

is clearly of dubious validity to extrapolate the results of animal experiments to human patients, however a number of the ideas discussed above may lend themselves to further investigation in man.

Recent improvements in immunocytochemistry and in situ hybridisation readily lend themselves to the study of histocompatibility antigen expression in metastasis and surrounding host tissues. These studies may be more meaningful if these are viewed in the context of cell interactions between tumour and host tissue, rather than the narrow considerations of host immunity which has dominated previous studies of this type.

The effect of various tissues on the growth of metastatic tumour cells could be investigated in a number of ways. Modern imaging technique could be used to monitor the growth rate of deposits at a variety of sites in the same patient. This would be of particular interest before and after excision of the primary tumour. Local recurrence after a long latent period is often seen, particularly in breast cancer. The application of the many available genetic and cell markers would show whether genetic or phenotypic changes had occurred. Finally, the study of human tissue growth factors is rapidly expanding. It is reasonable to expect that advances in this field will clarify many of the problems and provide mechanisms for the phenomena described above.

FIGURE 1

Specimen of stomach and duodenum showing extensive colonisation of the duodenal mucosa by metastatic malignant melanoma, but no evidence of spread to the adjacent gastric mucosa.

(Photograph courtesy of Prof. R.B. Goudie)

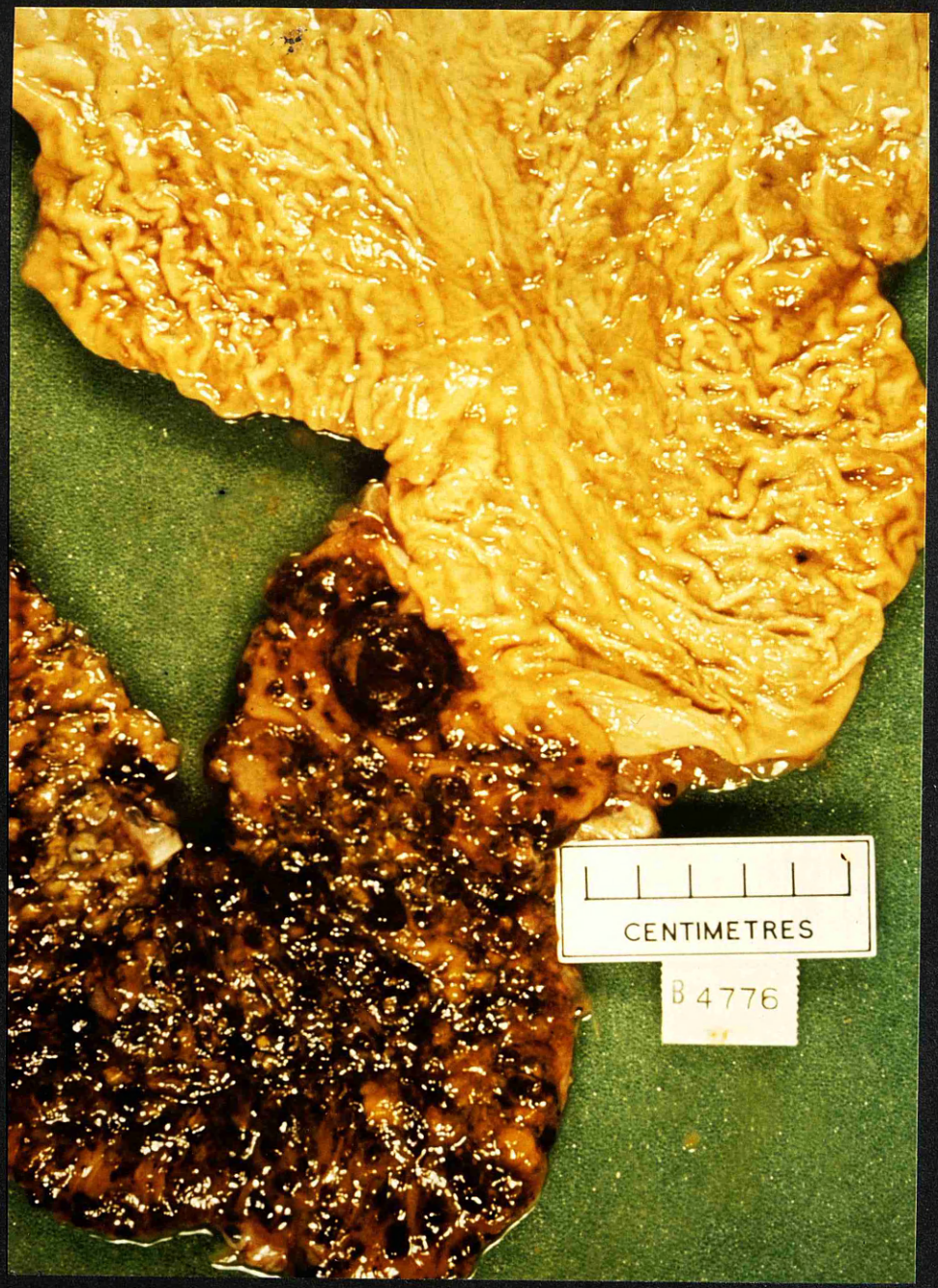

CENTIMETRES
B 4776

FIGURE 2

Typical growth curve for B16 F10 melanoma cells. The number of cells per 80cm^2 flask were counted and expressed as mean ± 1 standard error.

A similar growth curve was observed with B16 F1 cells.

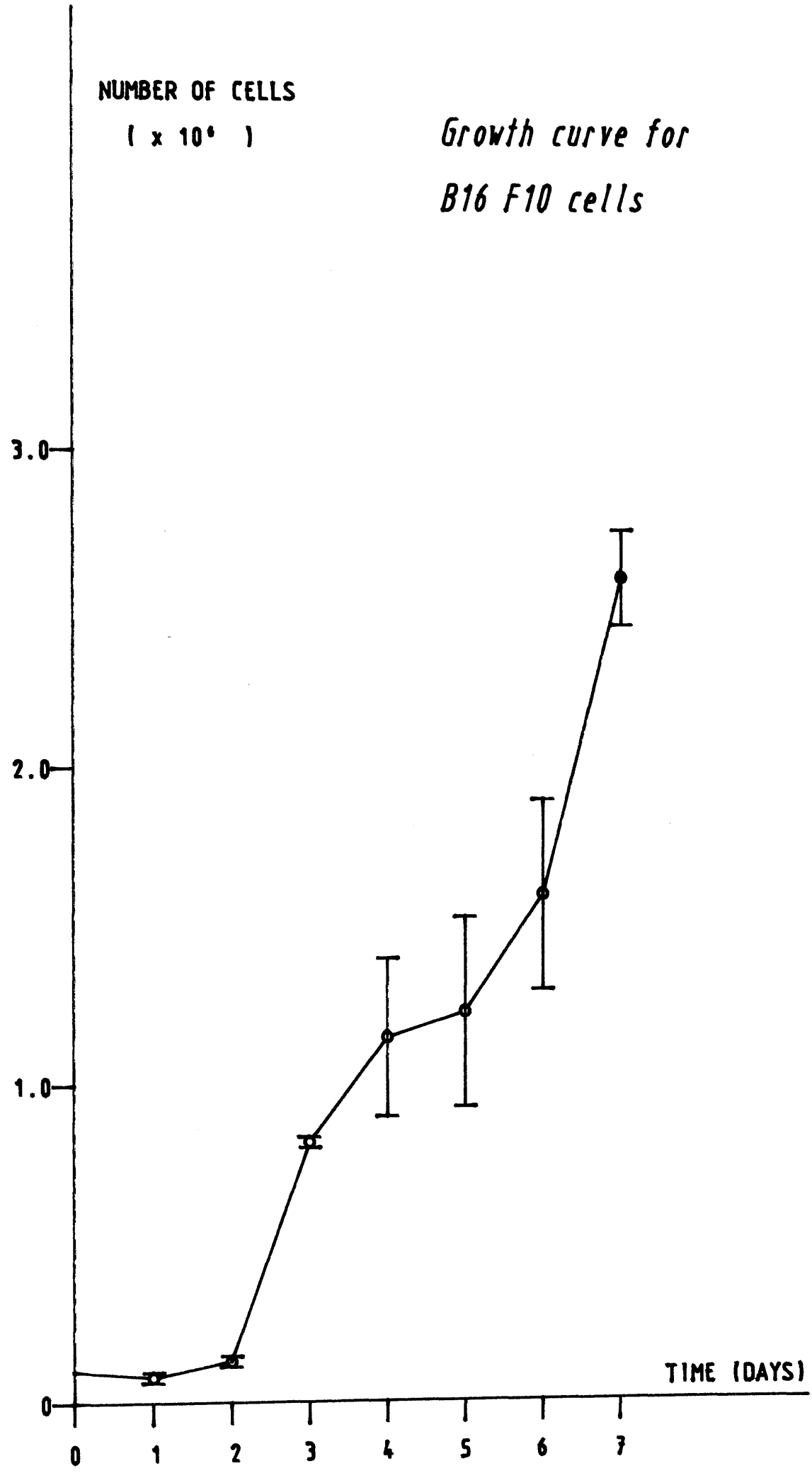

NUMBER OF CELLS
(x 10⁶)
Growth curve for
B16 F10 cells
3.0
2.0
1.0
0
TIME (DAYS)
0
1
2
3
4
5
6
7

FIGURE 3

Relationship between the number of tumours counted on the pleural surface of both lungs to the area occupied by tumour deposits, as determined by histomorphometry.

Best fitting line determined by linear regression analysis.

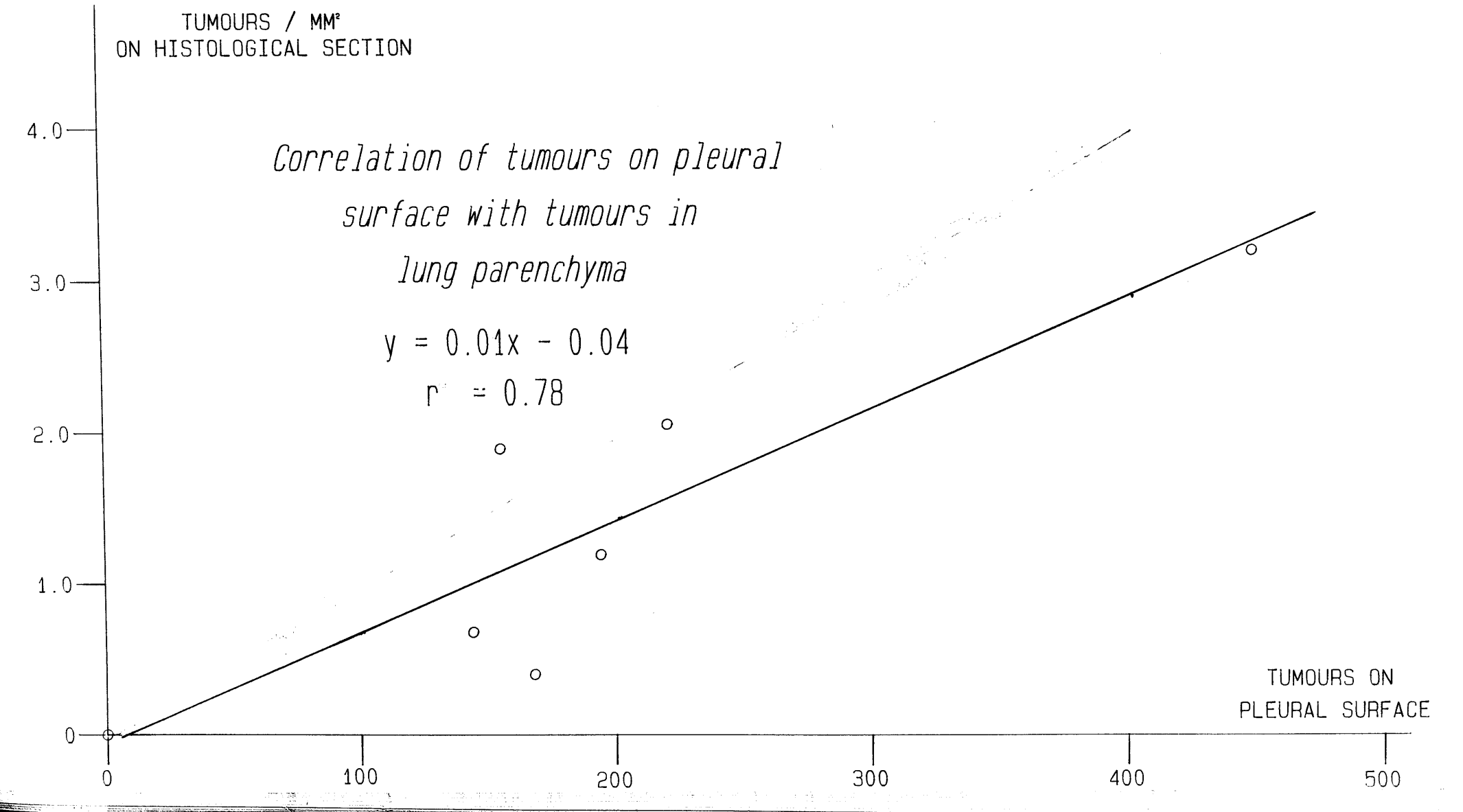
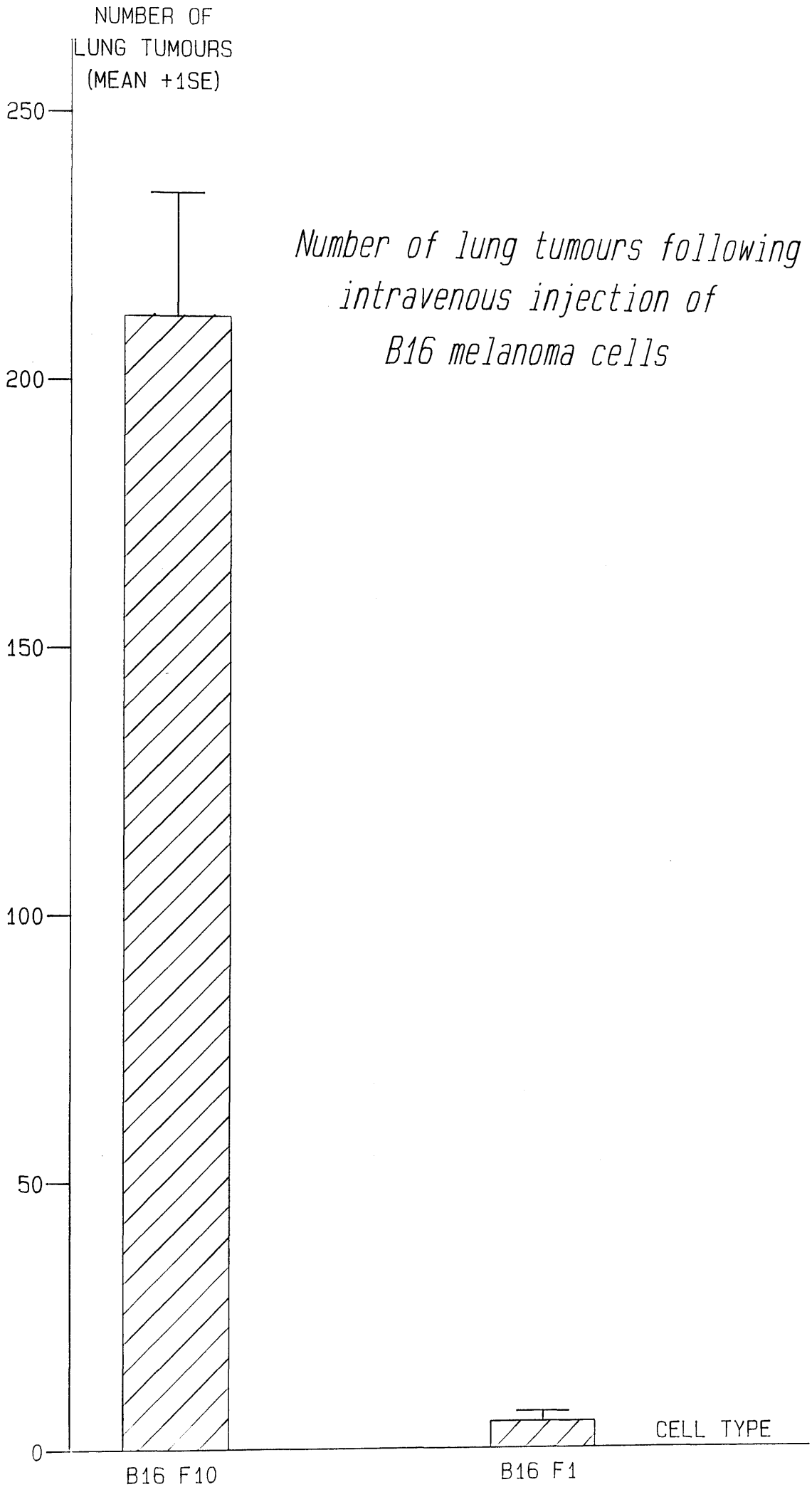

TUMOURS / MM²
ON HISTOLOGICAL SECTION
4.0
3.0
2.0
1.0
0
Correlation of tumours on pleural surface with tumours in lung parenchyma
y = 0.01x - 0.04
r = 0.78
TUMOURS ON PLEURAL SURFACE
0
100
200
300
400
500

FIGURE 4

The number of lung tumours (counted as visible pleural deposits) which resulted from the intravenous injection of 2×10^5 B16 F10 cells was significantly greater than the number of tumours which followed from the injection of the same number of B16 F1 cell ($p < 0.0001$).



Number of lung tumours following intravenous injection of B16 melanoma cells

FIGURE 5

Comparison of the number of tumours in the small intestinal mesentry and serosa following injection of B16 F10 and F1 cells. There is no significant difference in the number of tumours produced by each cell line.

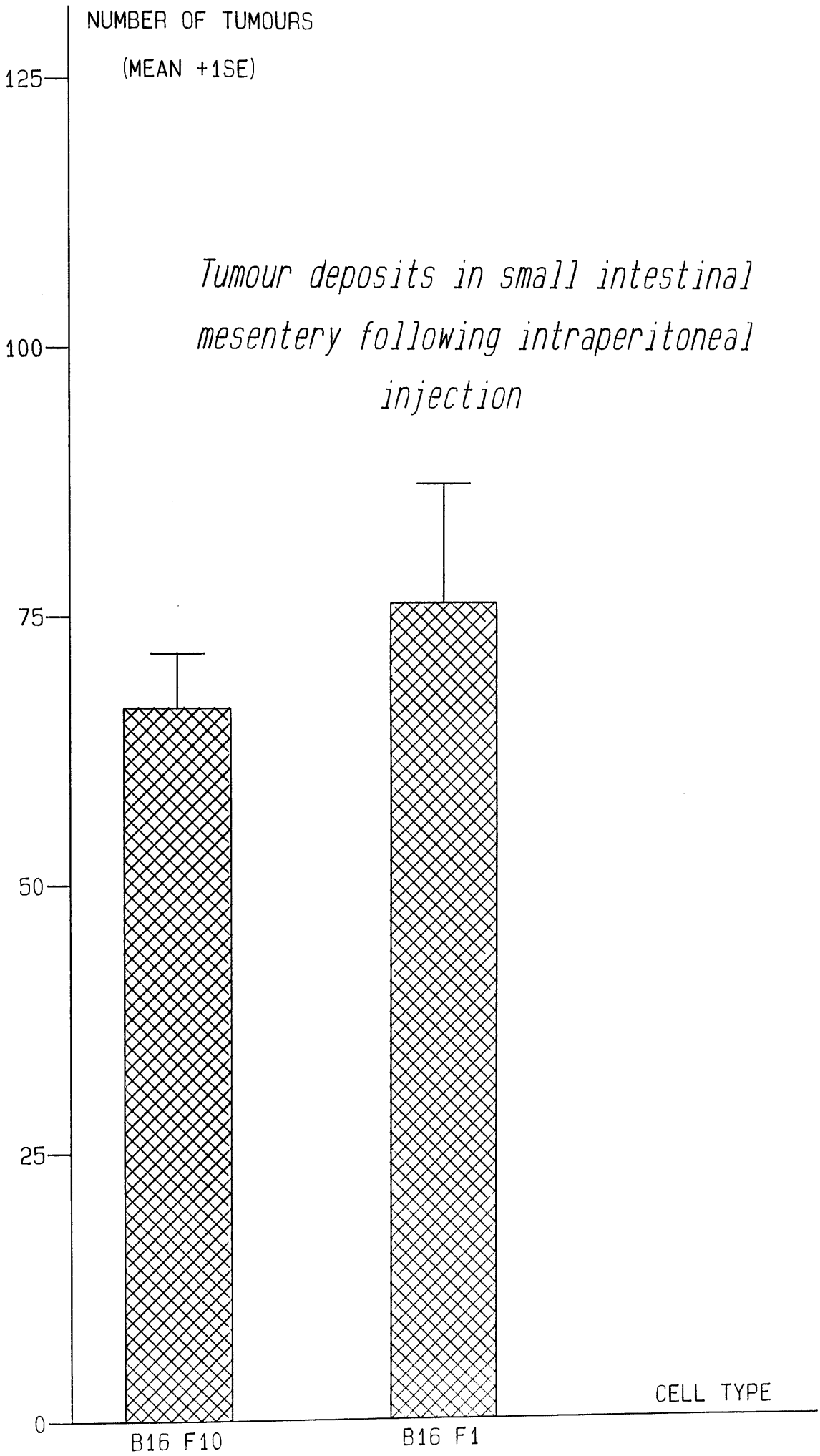

NUMBER OF TUMOURS
(MEAN +1SE)
125
100
75
50
25
0
Tumour deposits in small intestinal mesentery following intraperitoneal injection
CELL TYPE
B16 F10
B16 F1

FIGURE 6

Comparison of number of lung tumours (± 1 SE) produced by intravenous injection of B16 F10 and F10 cells which had been passaged in the peritoneal cavity eighteen times (F10 P18). The difference in the mean number of tumours is not statistically significant.

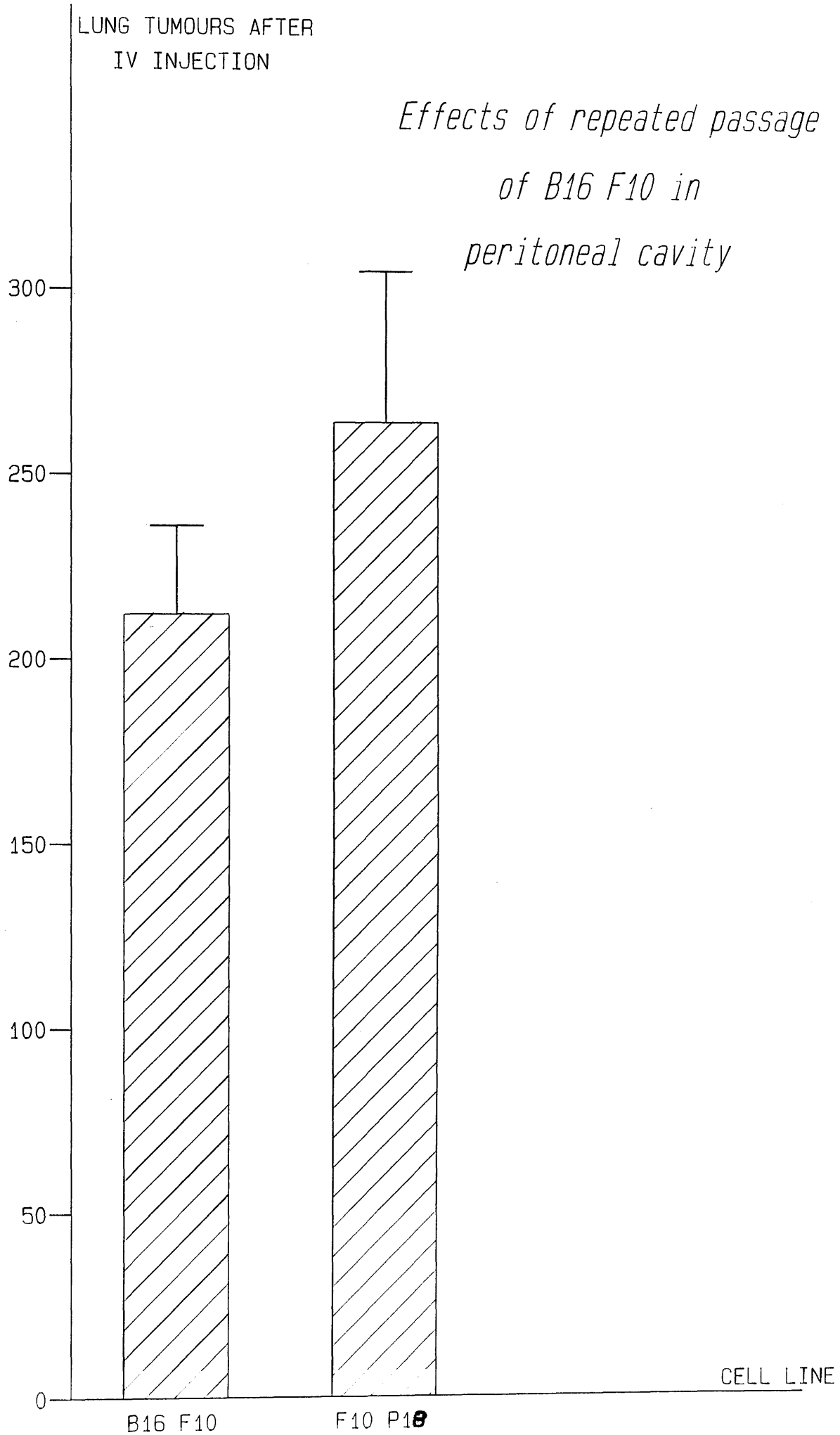

LUNG TUMOURS AFTER
IV INJECTION
Effects of repeated passage
of B16 F10 in
peritoneal cavity
300
250
200
150
100
50
0
B16 F10
F10 P18
CELL LINE

FIGURE 7

Comparison of the number of mesenteric deposits produced by intraperitoneal injection of B16 F1 (red) cells and F1 passaged 9 times (blue) and 16 times (green) through the peritoneal cavity.

Both passaged cell lines formed significantly fewer tumours than the unpassaged cell line ($p < 0.01$).

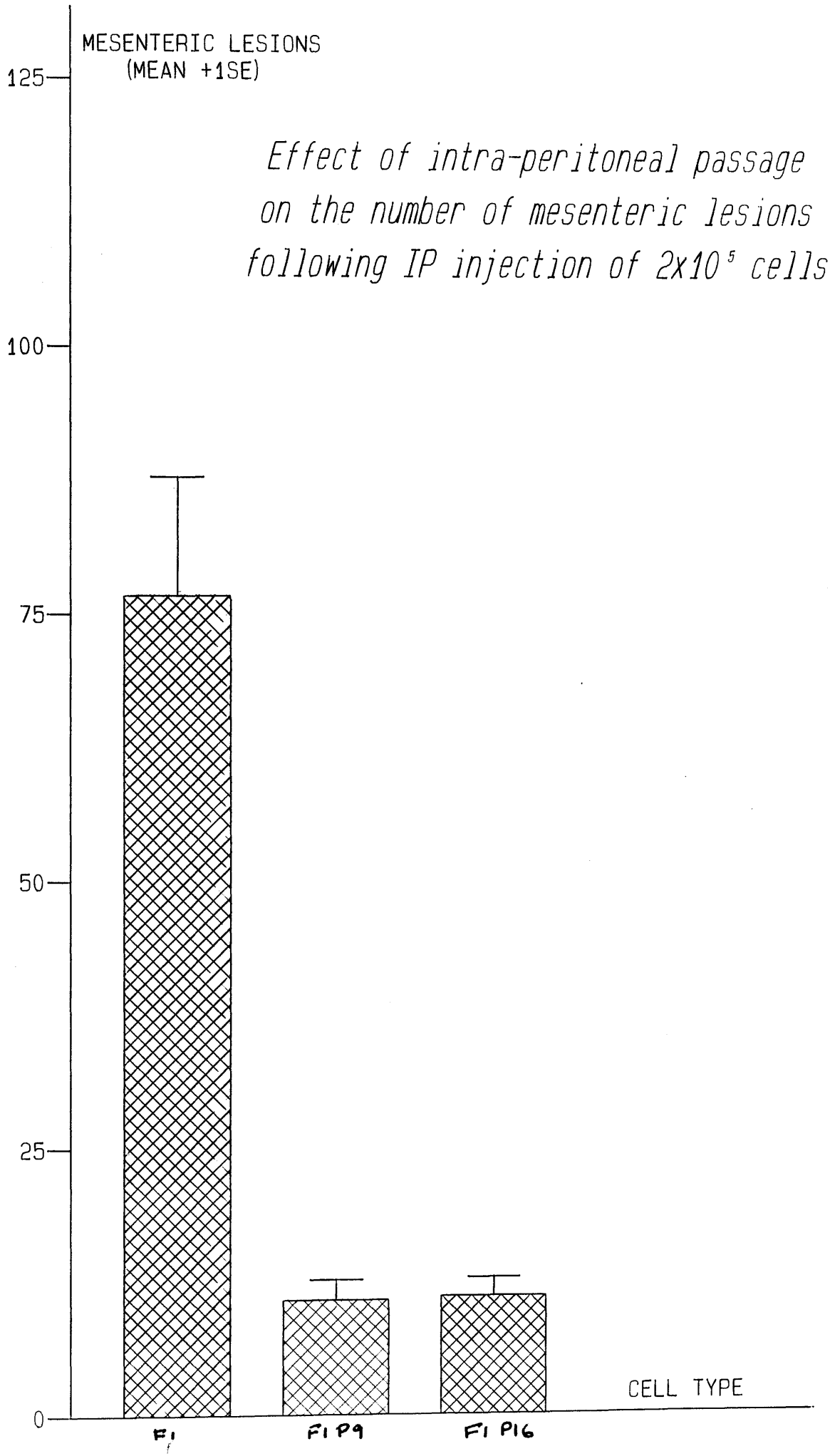

MESENTERIC LESIONS
(MEAN +1SE)
125
100
75
50
25
0
Effect of intra-peritoneal passage
on the number of mesenteric lesions
following IP injection of 2x10⁵ cells
CELL TYPE
F1
F1 P9
F1 P16

FIGURE 8

Cultures of B16 F10 cells were labelled with $Na^{51}Cr$. The medium was then replaced with non-radioactive medium and the retained radioactivity measured at various times.

From an experimental regression curve the half life of retention was estimated at 12.5 hours.

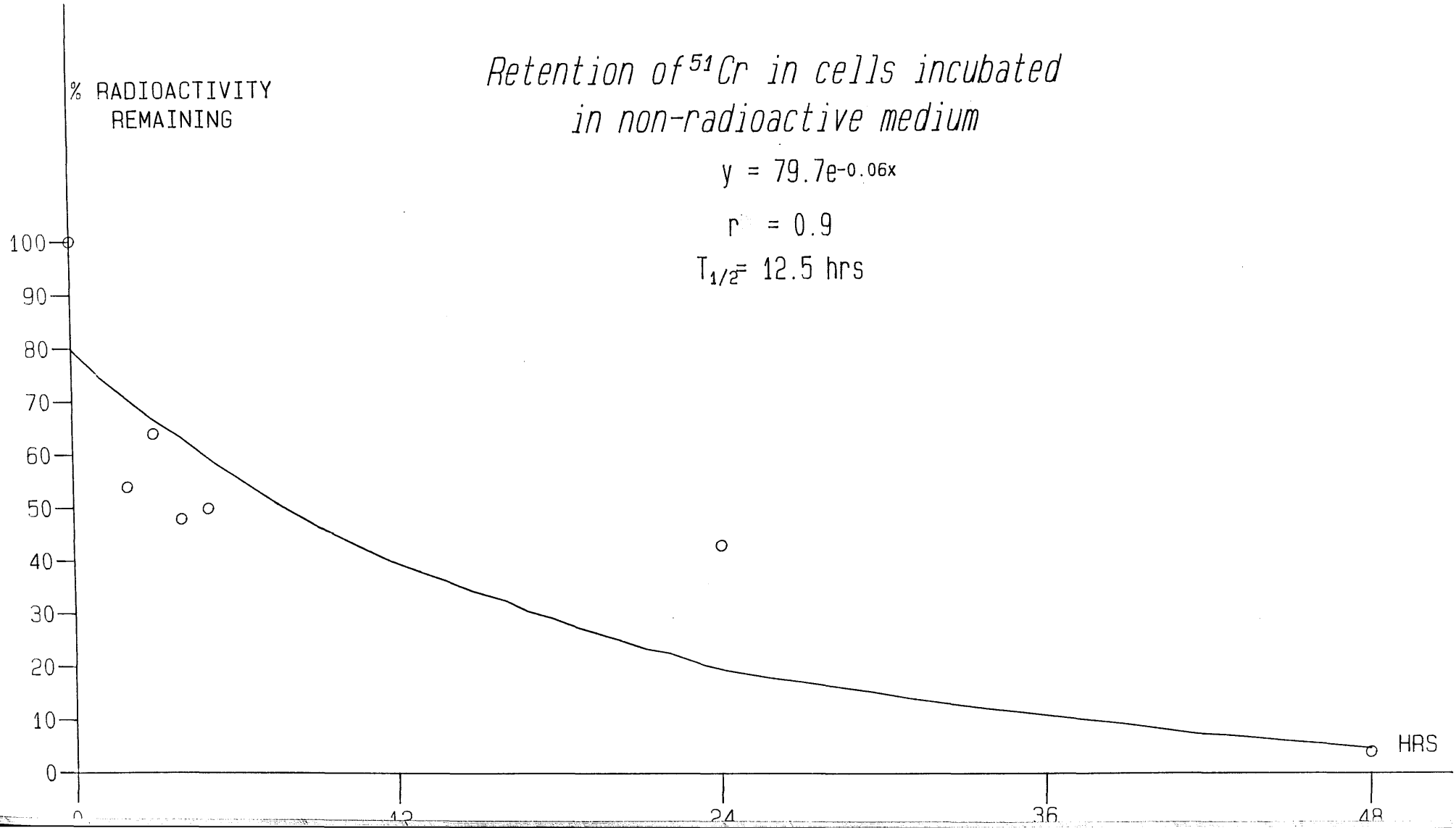

Retention of ^{51}Cr in cells incubated in non-radioactive medium
y = 79.7e-0.06x
r = 0.9
$T_{1/2}$ = 12.5 hrs
% RADIOACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
HRS

FIGURE 9

Comparison of mean (± 1 SE) radioactivity in the lungs of animals injected with ^{51}Cr labelled F10 and F1 cells. The measurements were made two hours after injection.

Significantly more F10 cells were trapped in the lungs than F1 cells ($p < 0.0001$).

The results are corrected to account for the efficiency of cell labelling as described in the text.

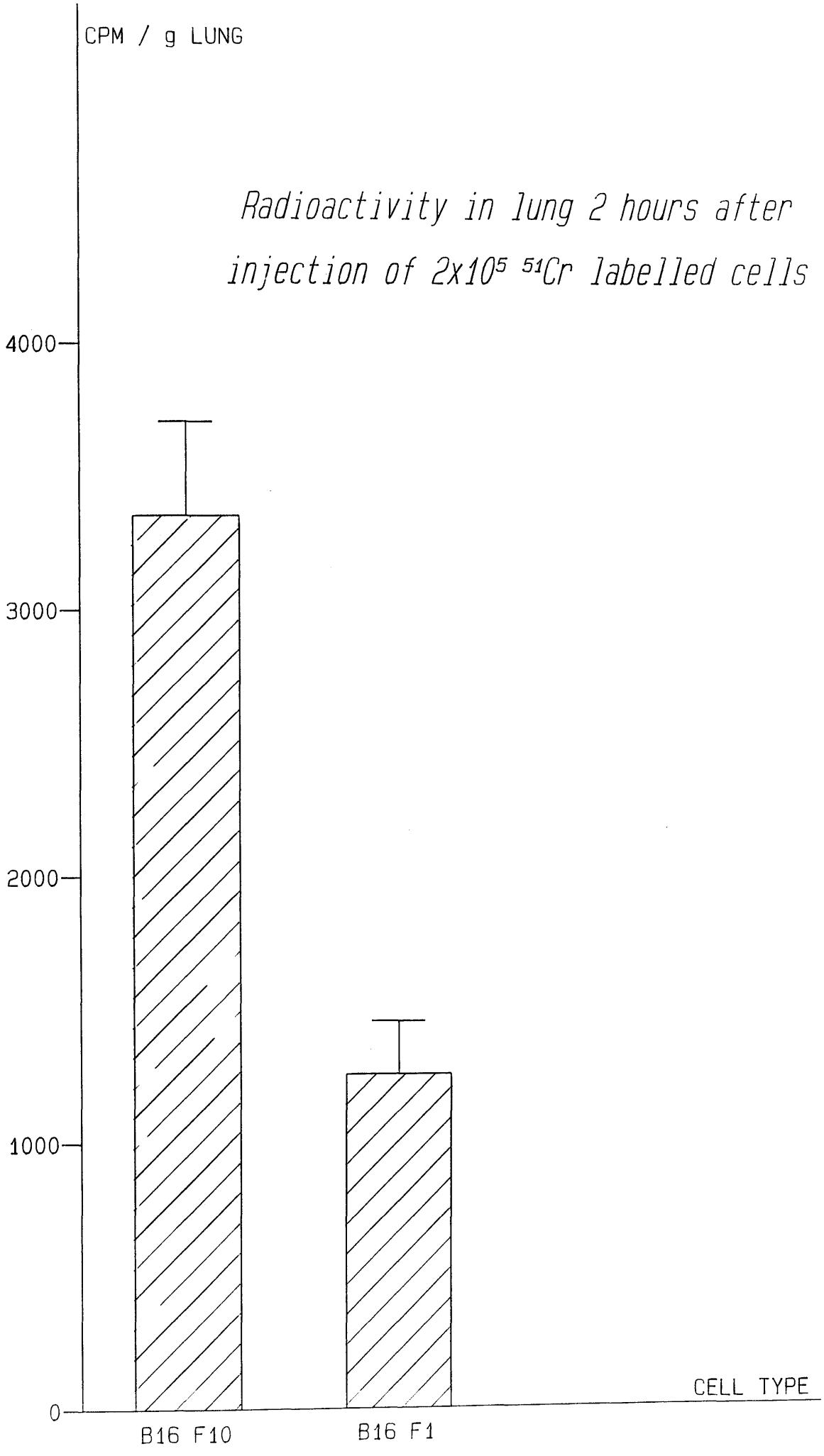

CPM / g LUNG
Radioactivity in lung 2 hours after injection of 2x10⁵ ⁵¹Cr labelled cells
4000
3000
2000
1000
0
B16 F10
B16 F1
CELL TYPE

FIGURE 10

Comparison of retention of radioactively labelled B16 F10 and F1 cells four hours after injection. There is significantly higher mean radioactivity in the lungs of animals injected with F10 cells ($p < 0.05$).

Values quoted in $Cpmg^{-1}$ are corrected for efficiency of cell labelling.

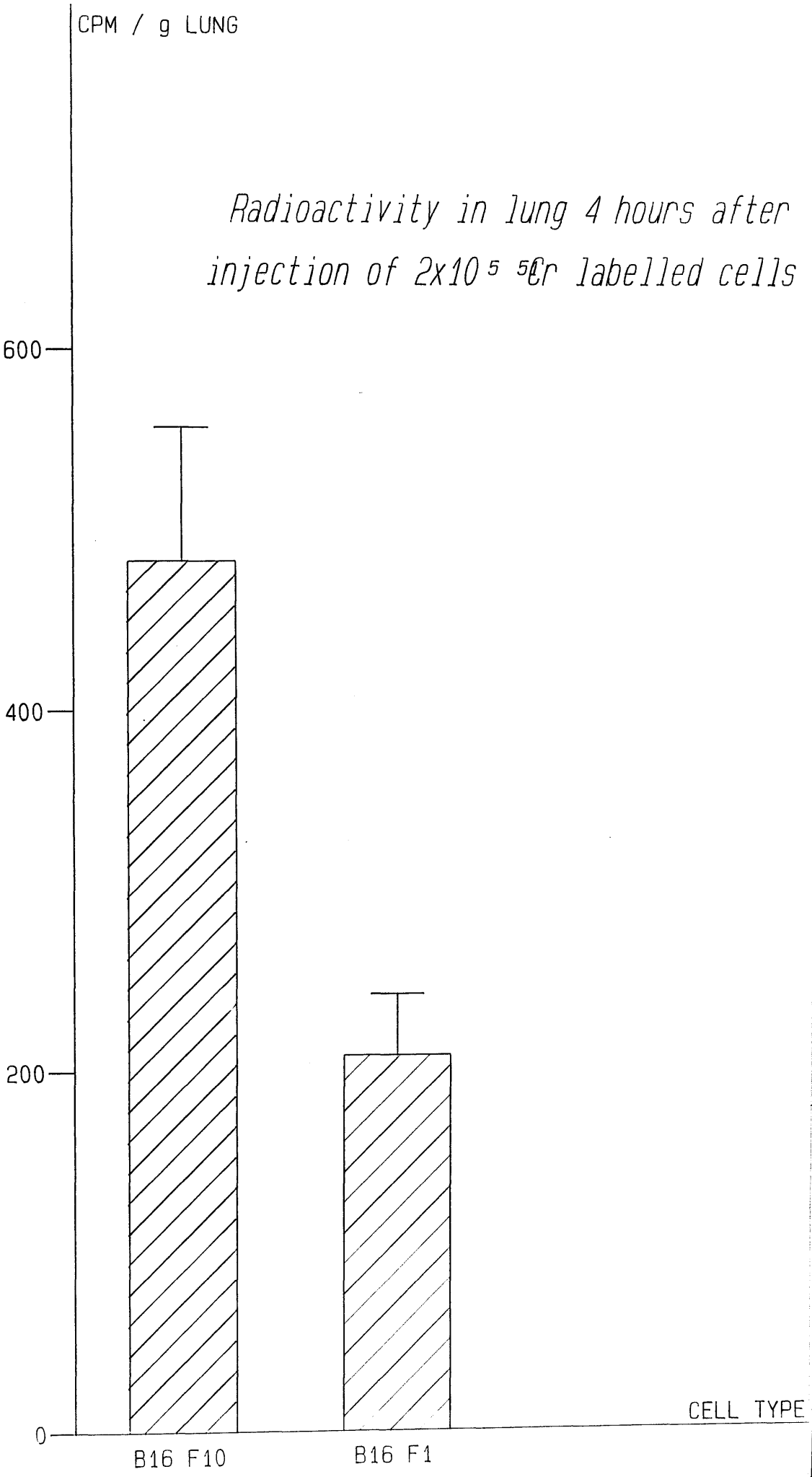

CPM / g LUNG
Radioactivity in lung 4 hours after injection of 2x10⁵ ⁵¹Cr labelled cells
600
400
200
0
B16 F10
B16 F1
CELL TYPE

FIGURE 11

Adhesion of Bl6 Fl0 cells (darkly staining) to cryostat section of mouse liver. H - E X 395.

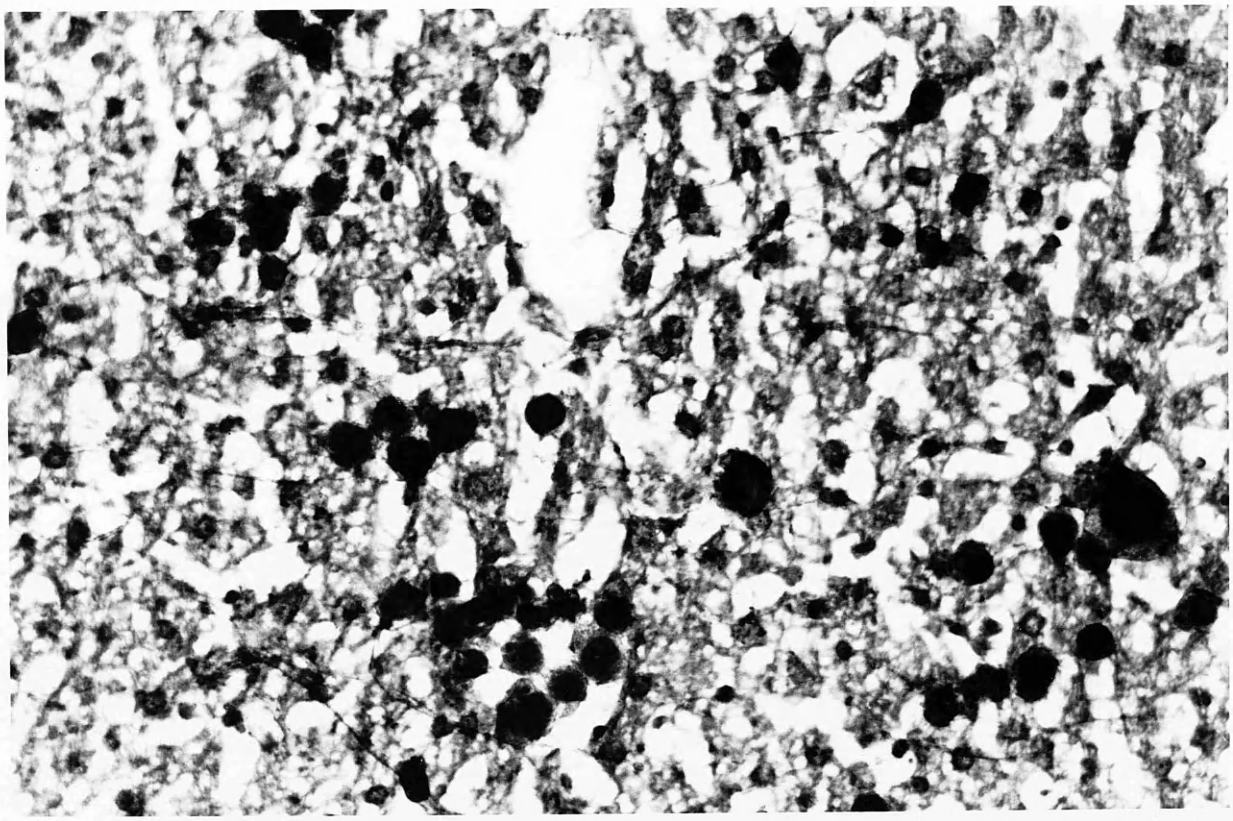
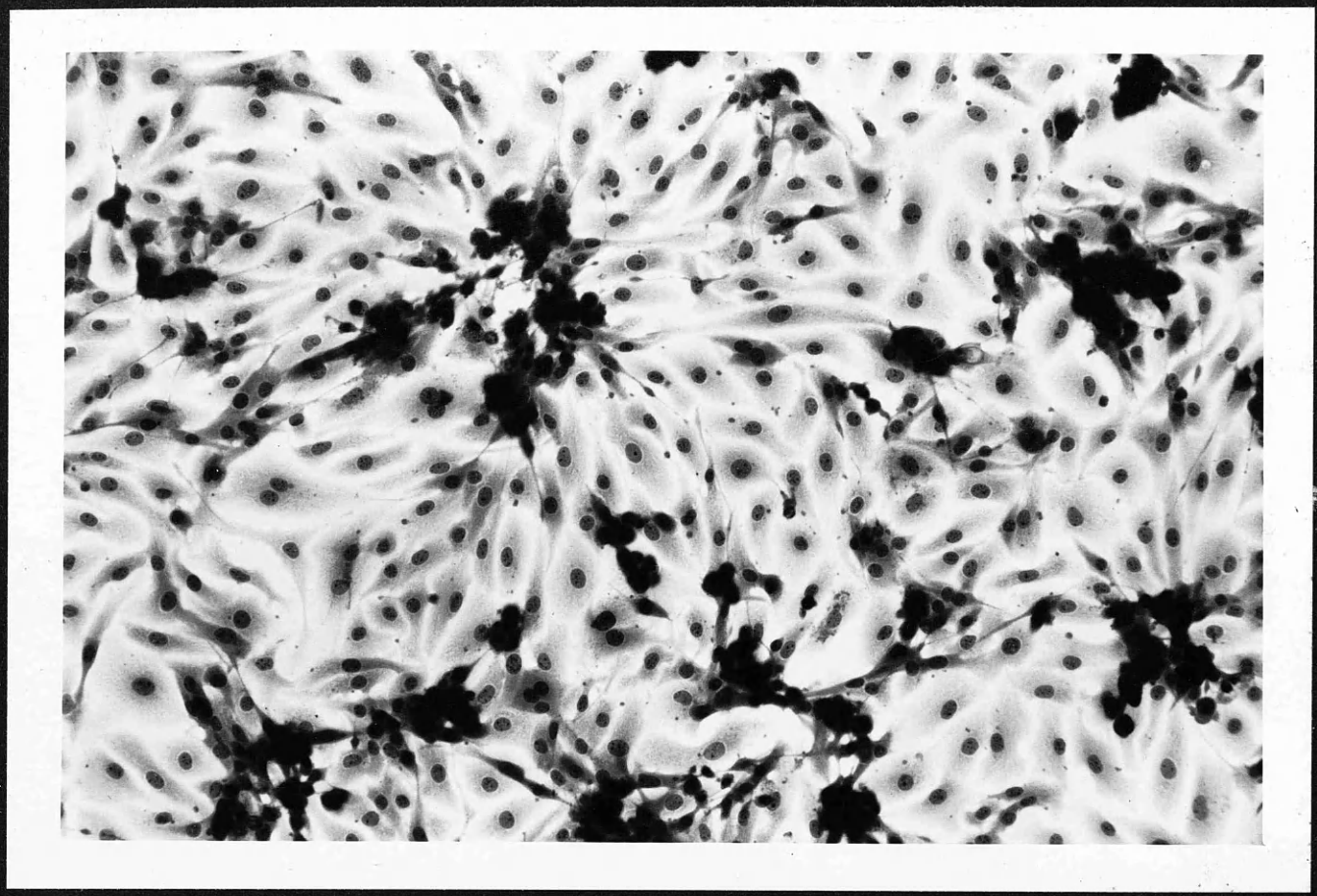


FIGURE 12

B 16 F10 cells grown on a coverslip. Most cells are spindle shaped and mononuclear. H & E x 155.

FIGURE 13

B16 F1 cells after seven passages in the peritoneal cavity. Increased number of multinucleated cells were present. H & E X 990.



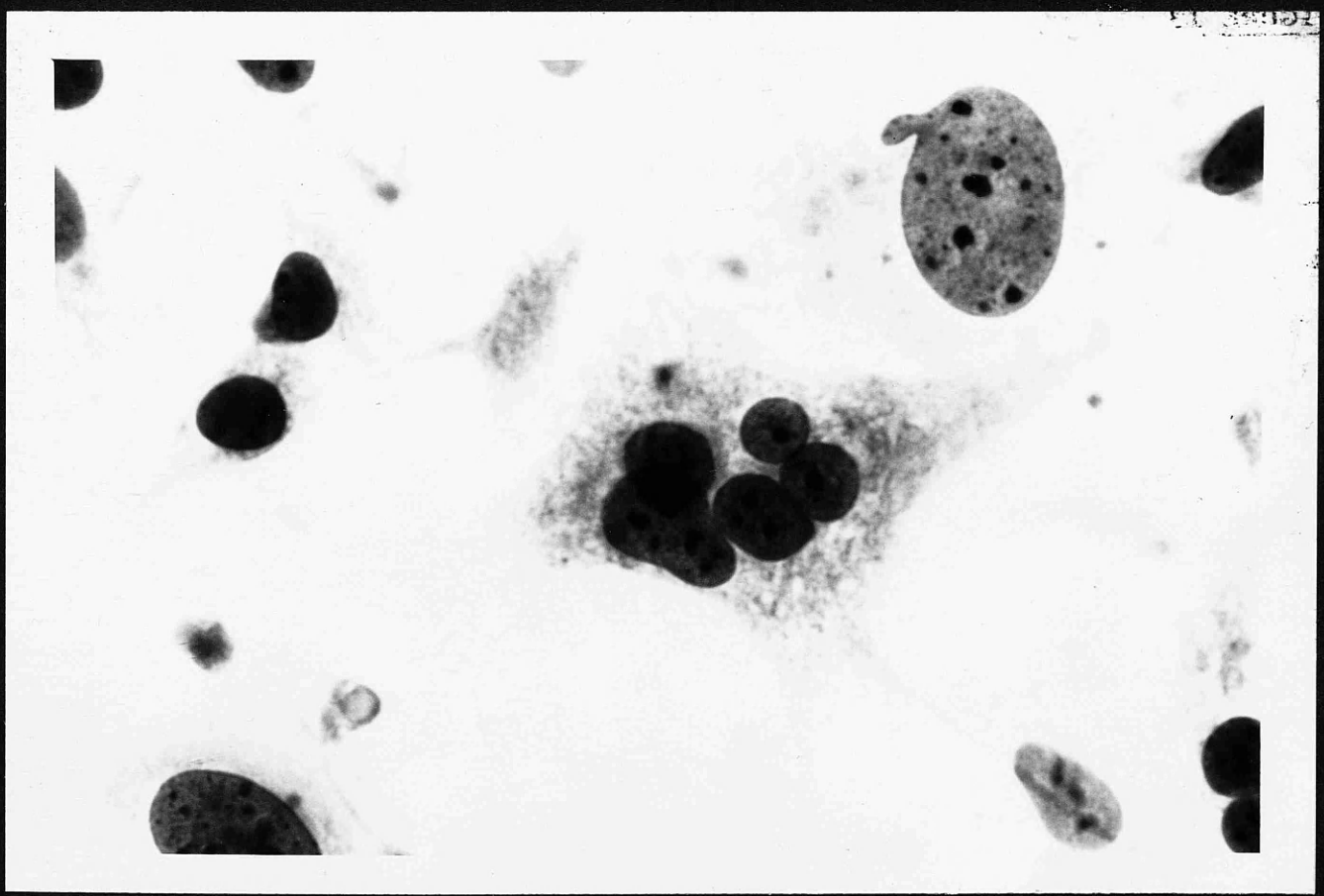


FIGURE 14

F1 cells incubated with 3,4 dihydroxy phenylalamine to demonstrate melanin synthesis. Reaction product is visible as dense cytoplasmic granules.

F10 and passaged cell lines also reacted positively with this reagent.

Dopa reaction X 784

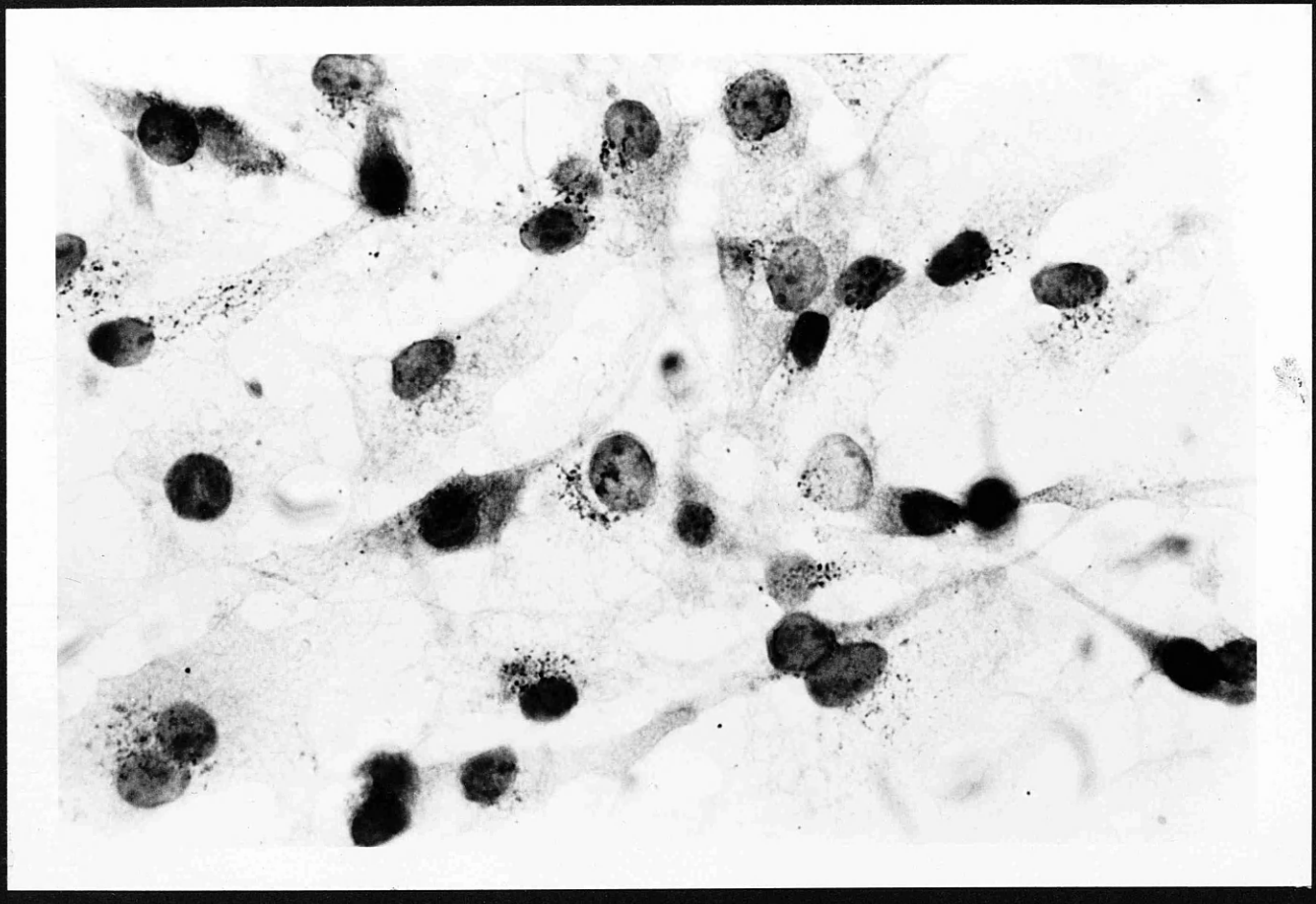
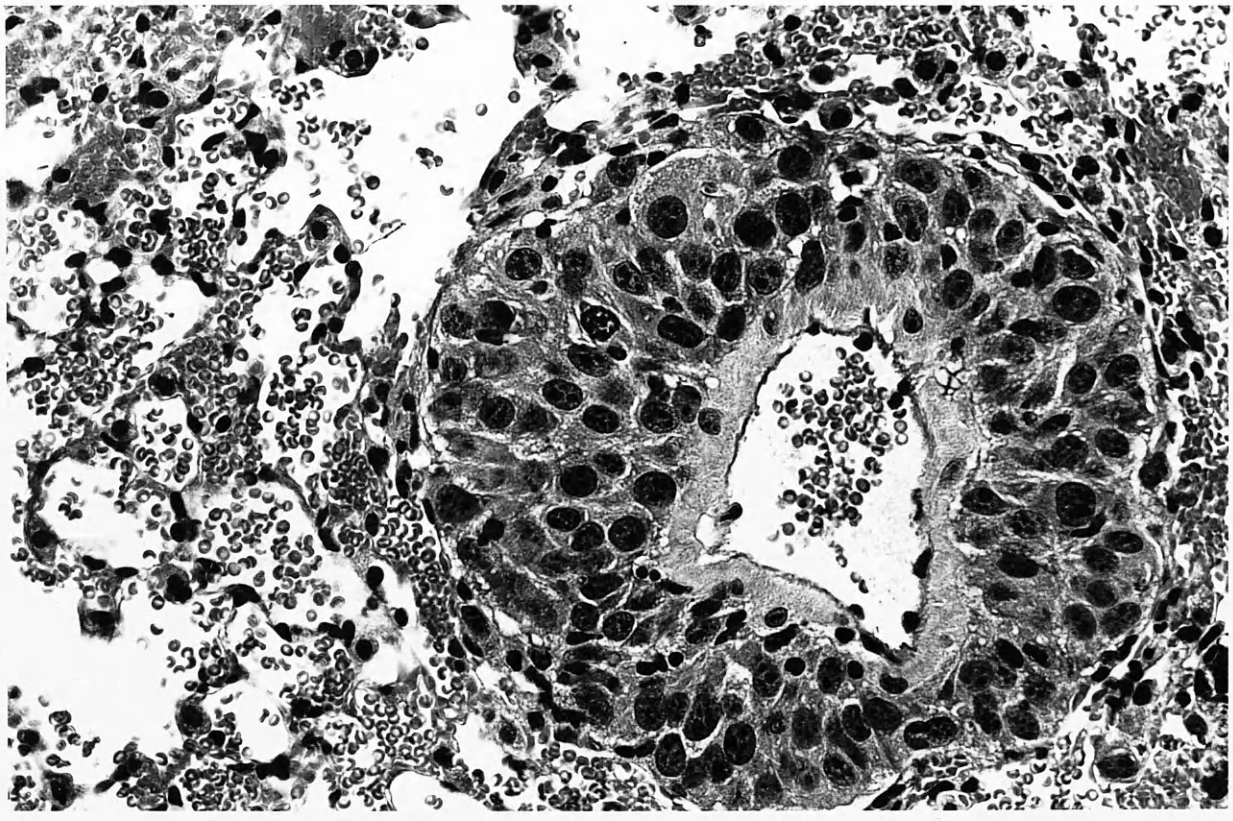


FIGURE 15

Section of lung from a mouse injected with B16 F10 cells. A metastatic deposit of melanoma is present surrounding a thin walled blood vessel. This was a commonly observed feature in lung deposits of this tumours. H & E x 395.

FIGURE 16

Section of lung showing deposit of B16 F10 cells growing under the pleural surface. H & E x 314.



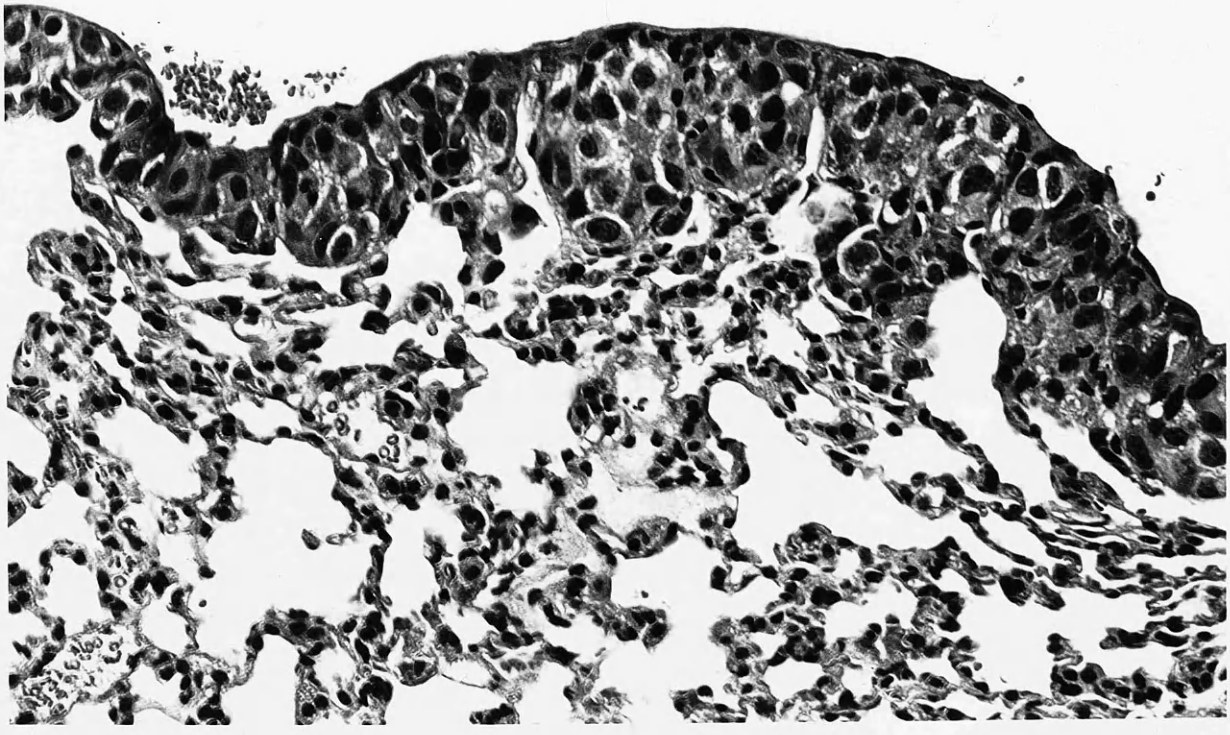
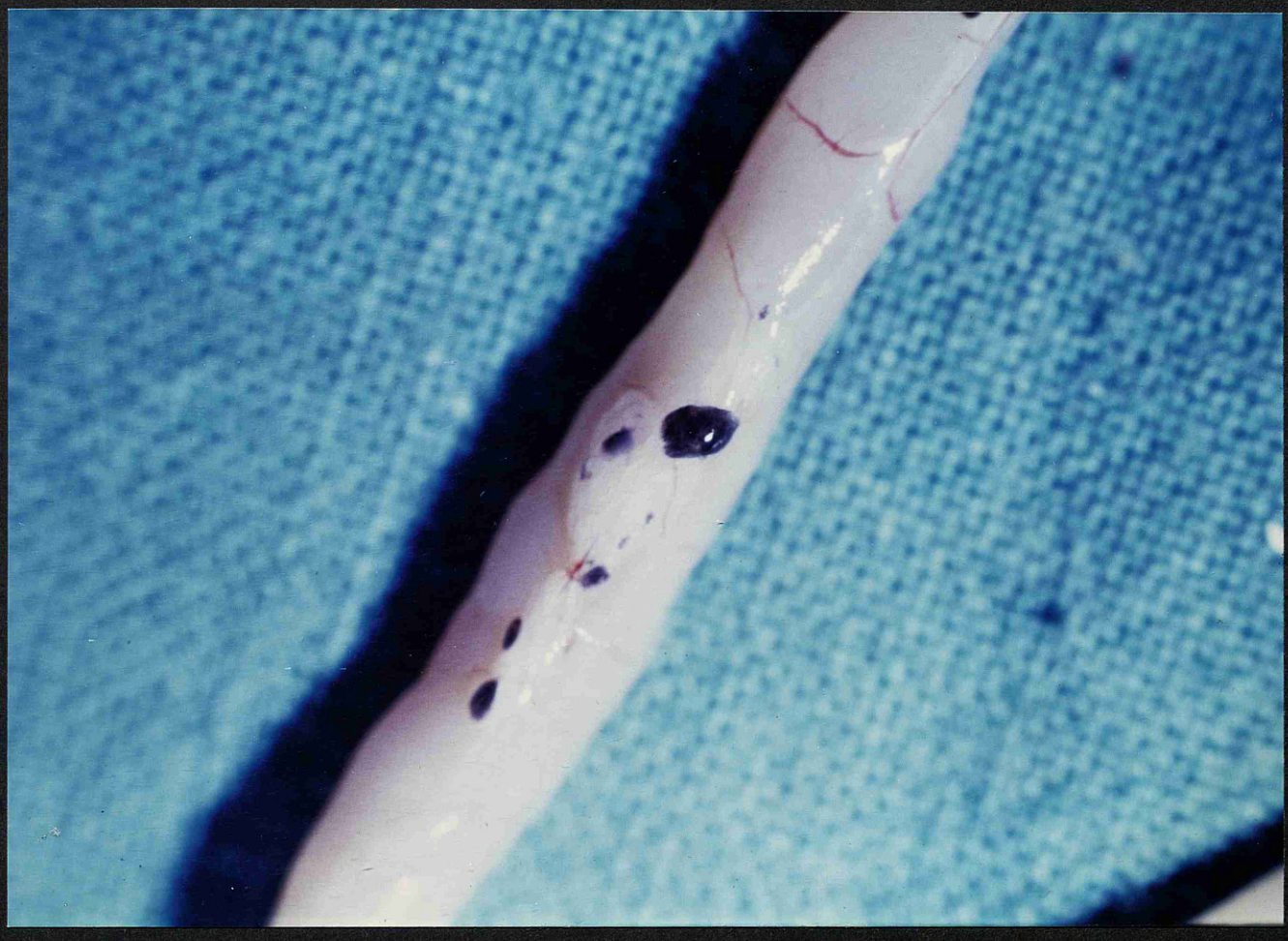


FIGURE 17

Deposits of B16 F1 melanoma growing on the serosa and mesentry of the small intestine following intraperitoneal injection.

FIGURE 18

Section of small intestine with adjacent deposit of B16 F10 melanoma growing in the serosal fat. H & E x 125.



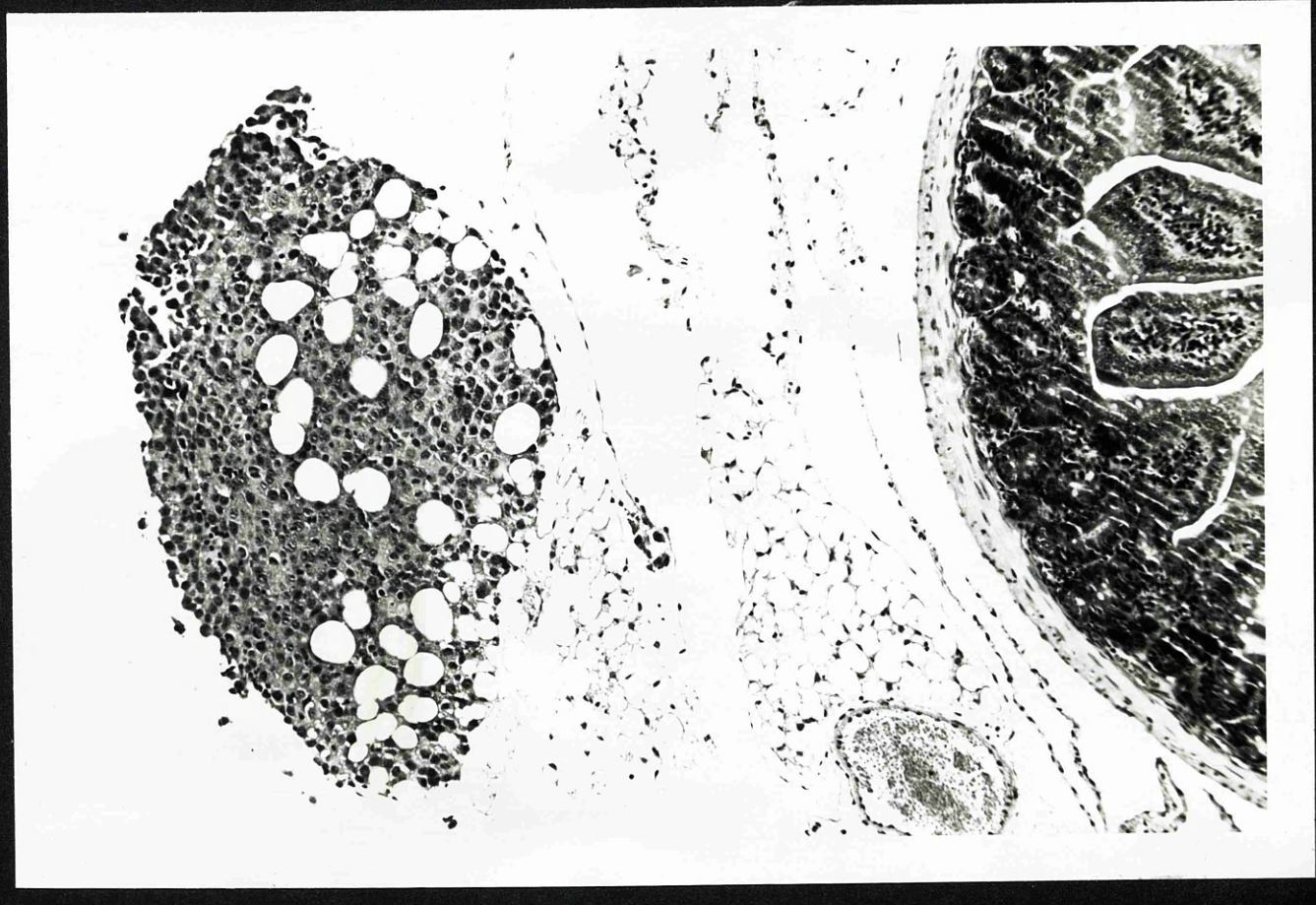


FIGURE 19

Mouse polyoma virus induced salivary gland tumour. This section shows a nodule of tumour separated from the adjacent normal gland by a thin fibrous capsule. There is no evidence of invasion.

A scattered lymphoid infiltrate is visible within the tumour. H & E x 190.

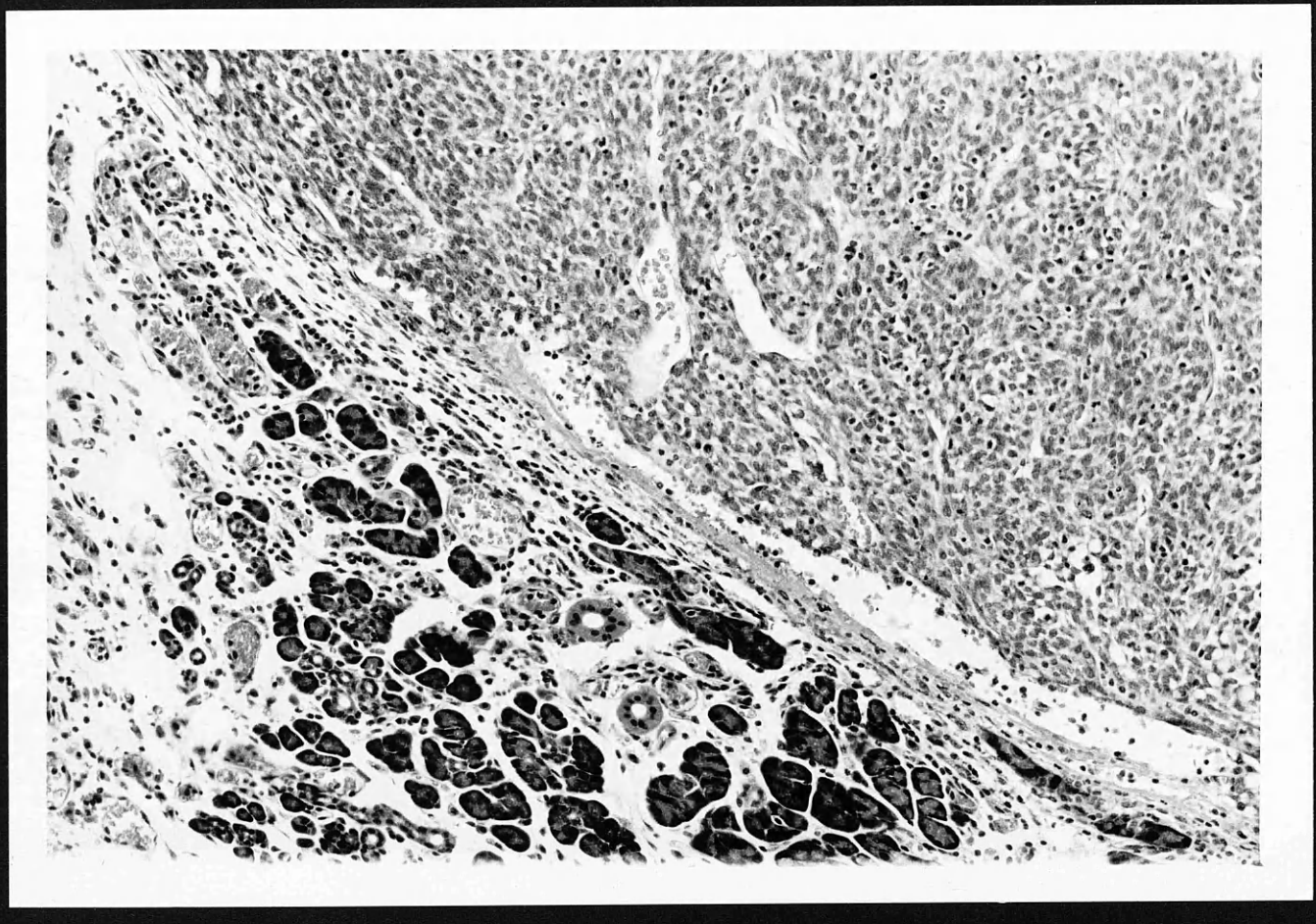
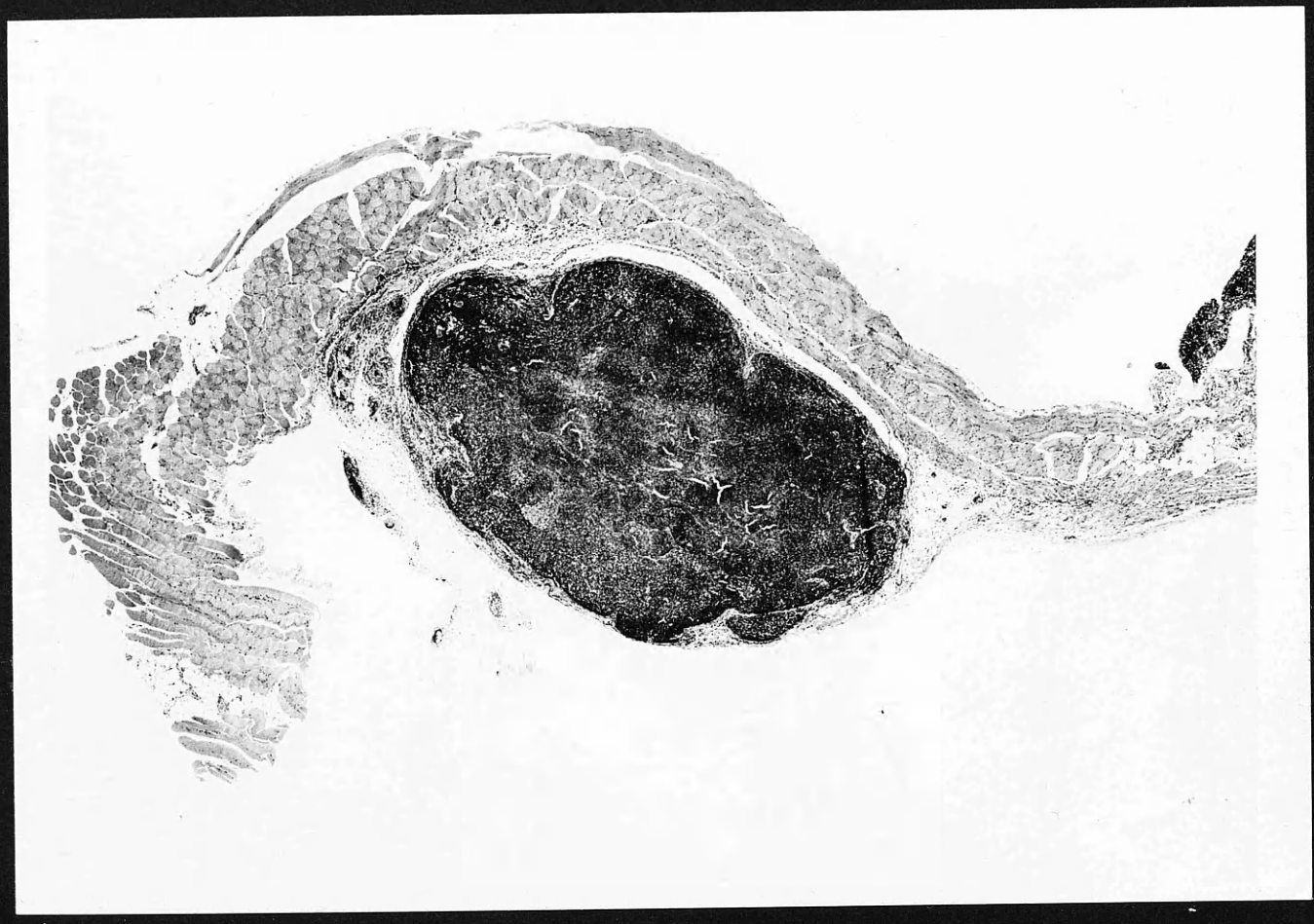


FIGURE 20

Section of peritoneum with attached deposit of mouse polyoma salivary tumour. The tumour was transplanted as a small cube of tissue four weeks previously. The tumour is attached by reactive fibrous tissue at its edge but shows little significant increase in size. H & E x 20.

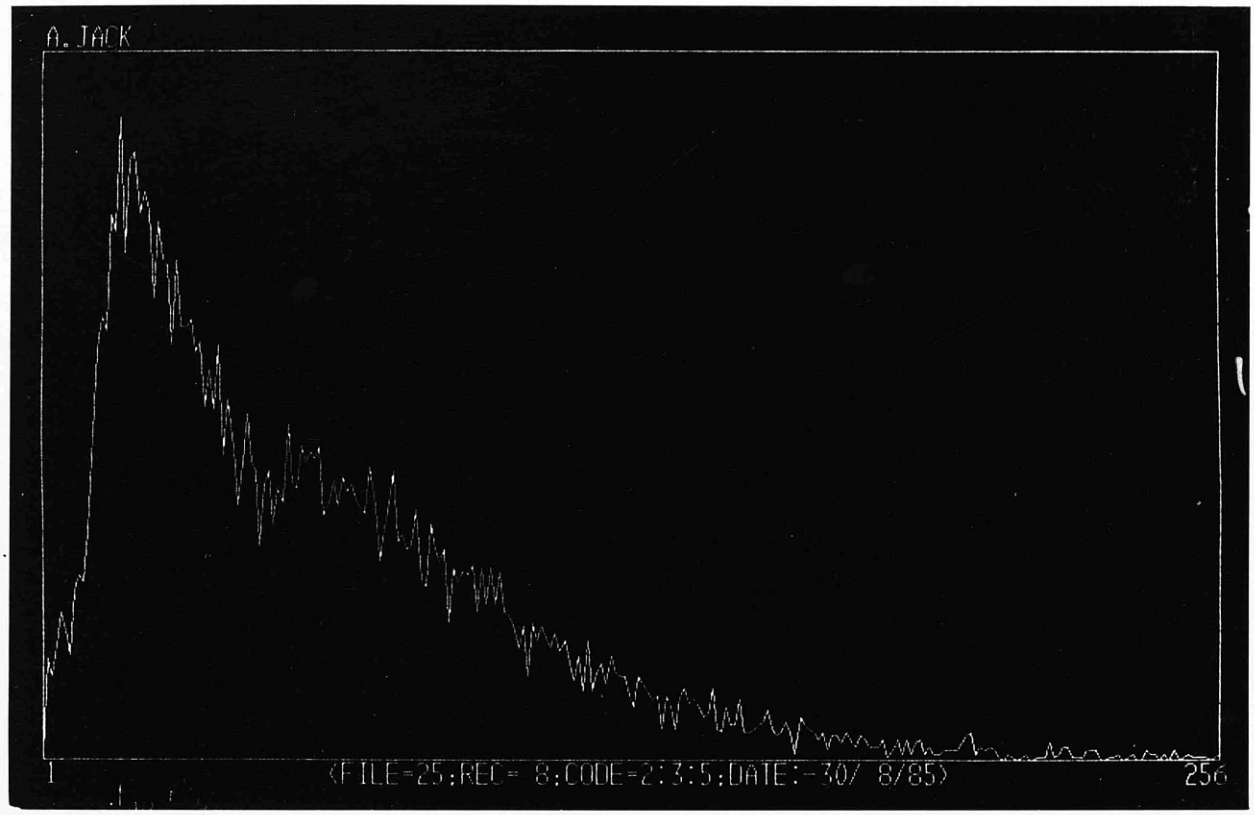


FIGURES 21 & 22

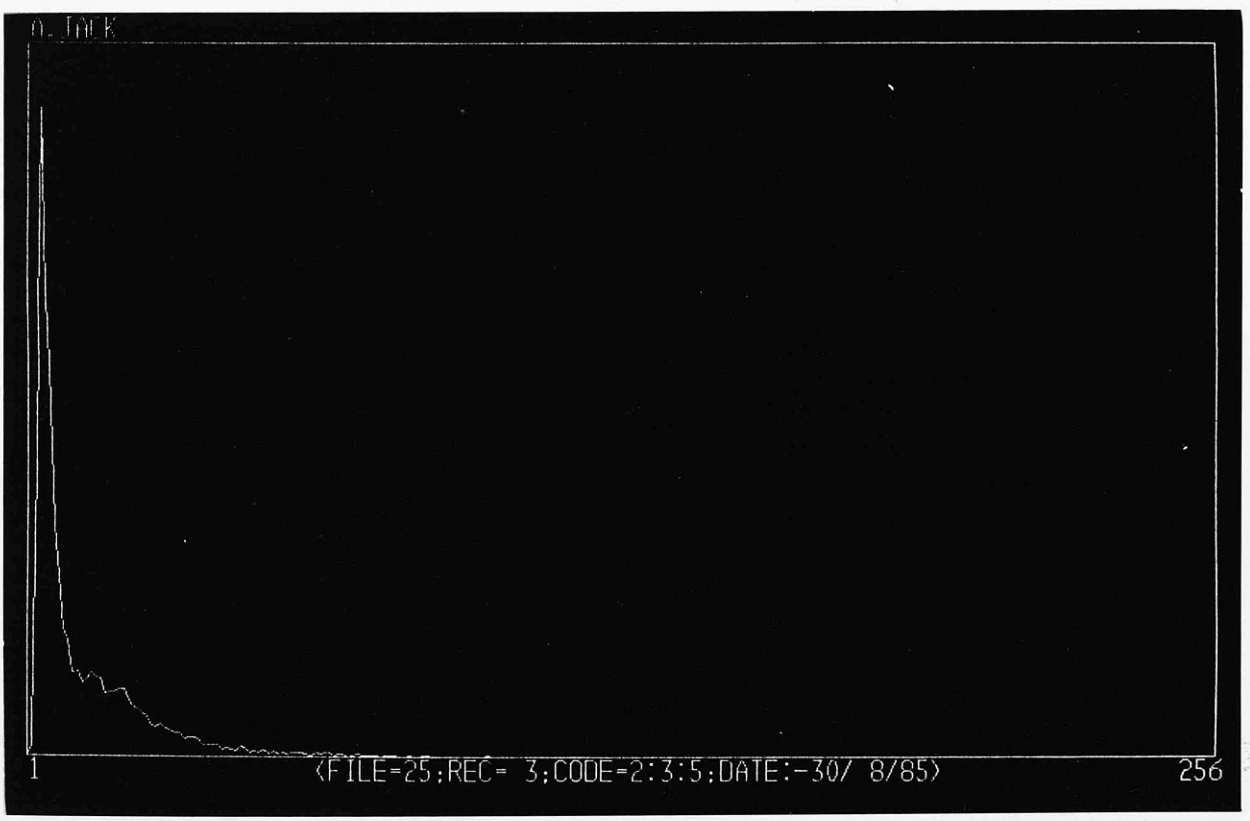
FACSII plots of number of cells (y-axis) and intensity of staining (x-axis). The intensity of fluorescence is measured in 256 steps.

Figure 21 shows a plot obtained from F10 cells stained with anti H-2^b(KD) Figure 22 shows a plot for F10 cells stained by the same method but omitting the specific antiserum. It is therefore a measure of cell autofluorescence and non-specific binding on rabbit anti-mouse immunoglobulin FITC conjugate.

NB: The y-axis scale on these diagrams is randomly selected by the microcomputer and is not comparable for Fig. 21 and 22. The same number of cells was counted in each case.


A. JACK
1
(FILE=25;REC= 8;CODE=2:3:5;DATE:-30/ 8/85)
256

21


A. JACK
1
(FILE=25;REC= 3;CODE=2:3:5;DATE:-30/ 8/85)
256

22

FIGURES 23, 24, 25

Plots of cell size (y-axis) versus fluorescence intensity.

Fig. 23: shows the size/intensity relationship for B16 F10 cells stained with anti-H-2(KD). There is no apparent correlation between size and intensity.

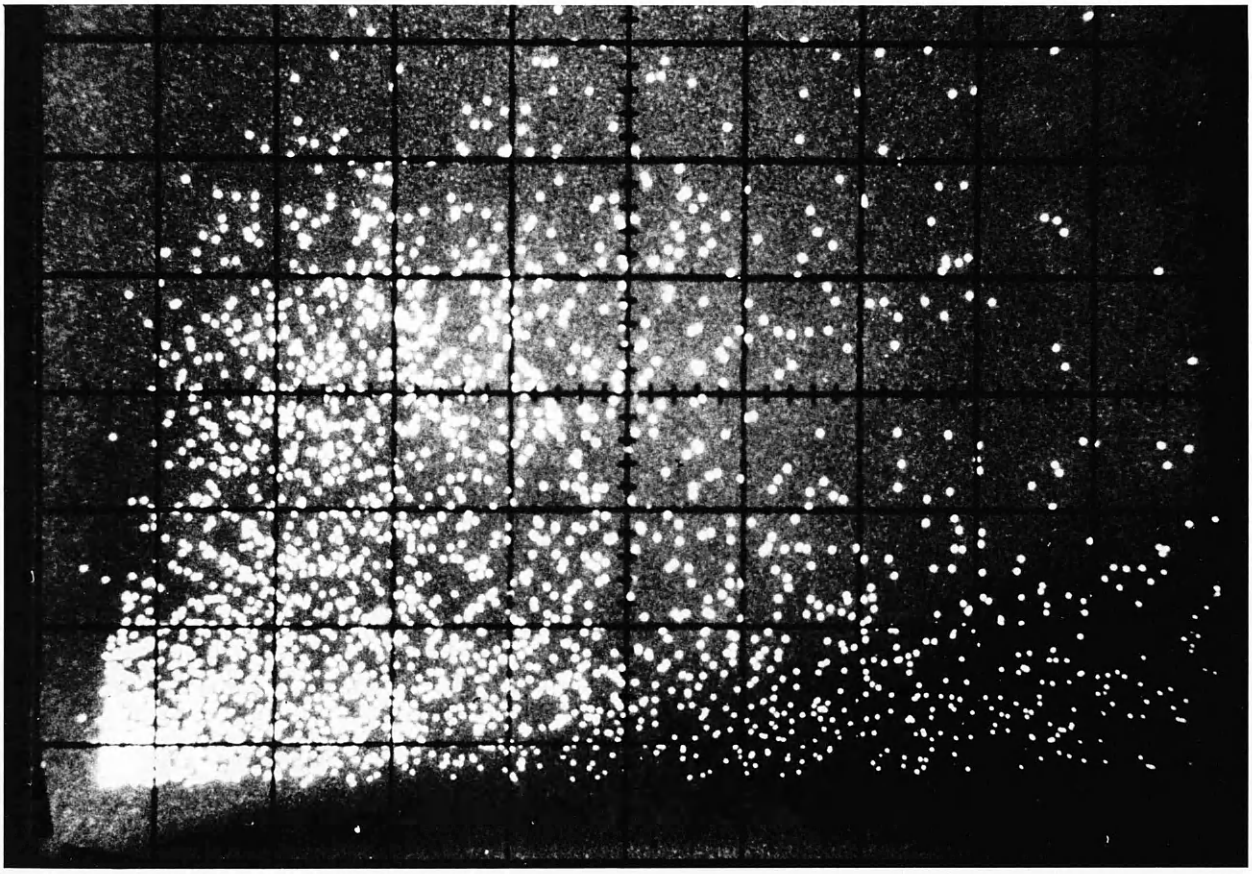


FIGURE 24

Fig. 24 shows the size/intensity relationship for B16 F1 cells stained with anti-1-A. The vertical band represents unstained cells. The near horizontal band are cells stained with the antibody. Again there is no relationship between size and intensity.

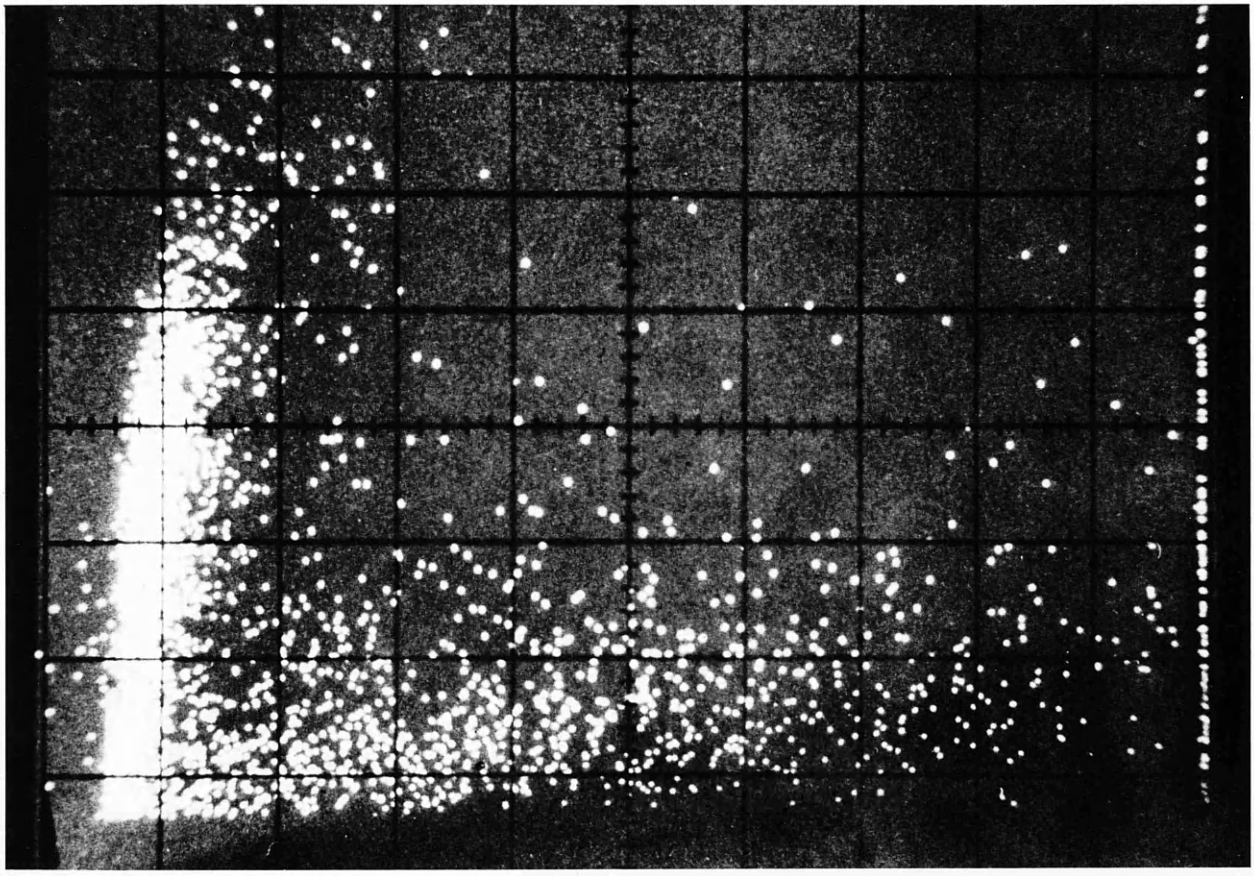


FIGURE 25

Fig. 25: shows a similar plot for cells stained by the same procedure omitting the primary antisera.

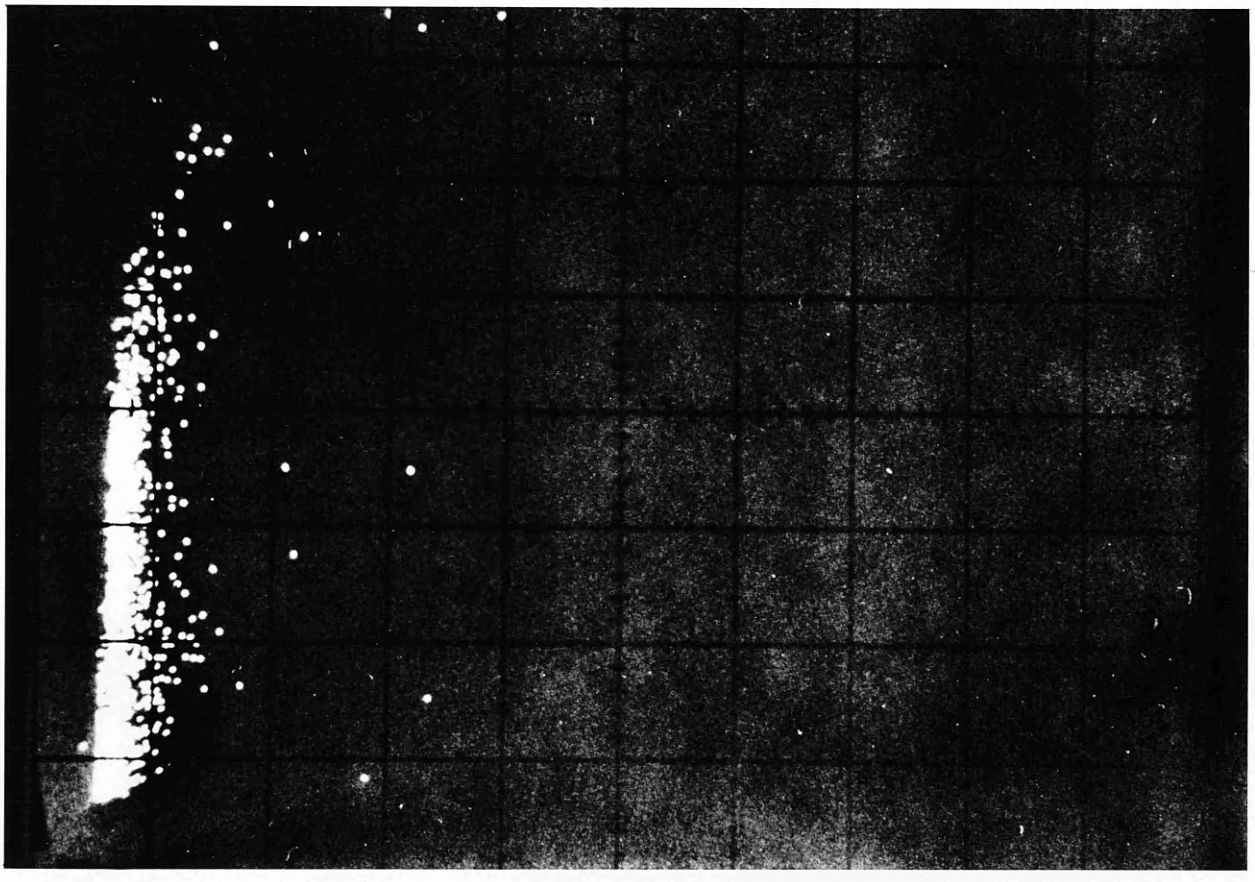
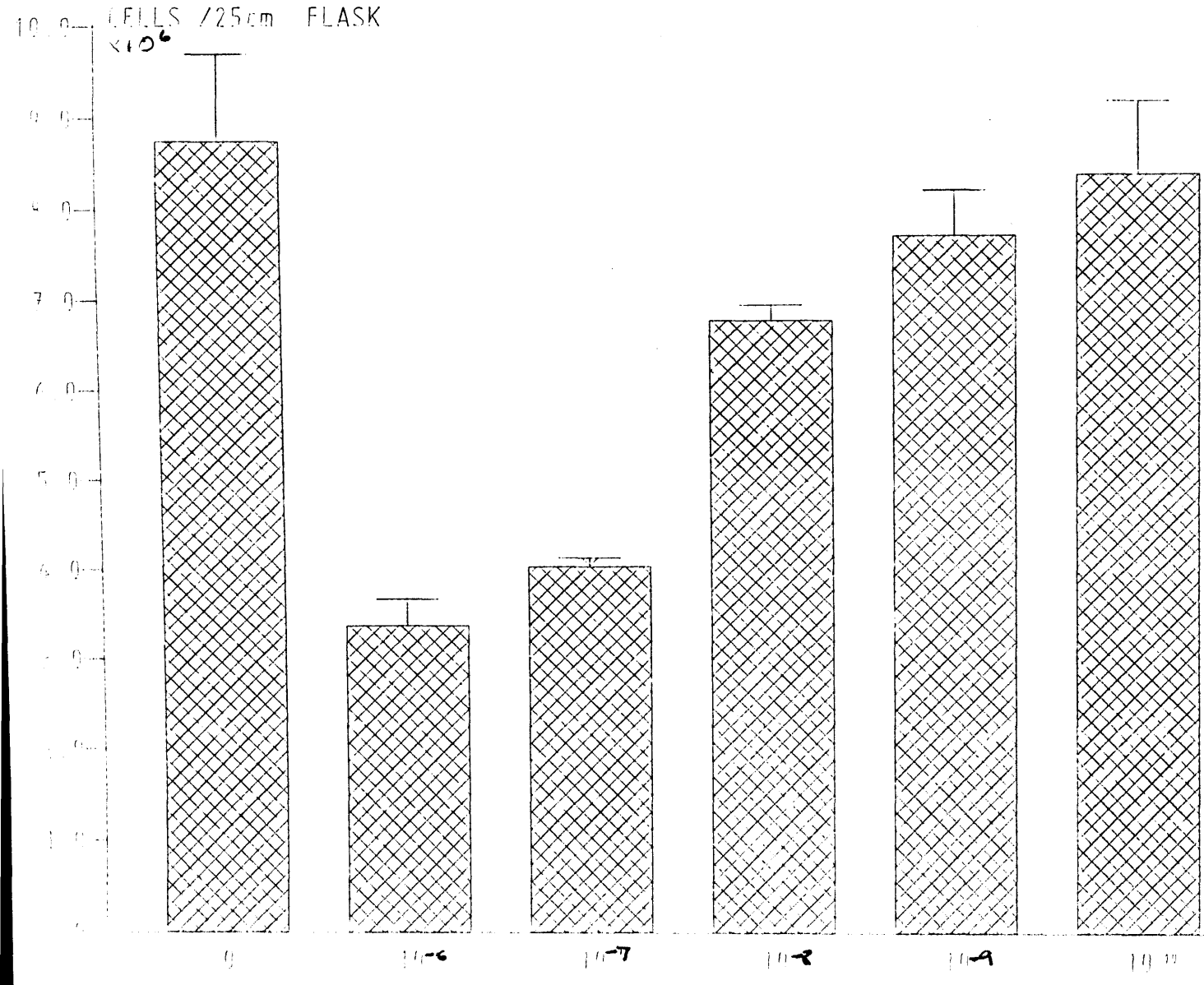


FIGURE 26

Graph showing the effect on cell growth of of B16 F10 cells with varying concentrations of retinoic acid. At concentrations of 10^{-6} - 10^{-8}M retinoic acid there was significant impairment of growth as compared to untreated control cells.



Cell growth after 5 days incubation with varying concentrations of Retinoic Acid

INITIAL CONCENTRATION :
3×10^5 CELLS /25cm² FLASK

FIGURE 27 - 32

These histograms show the effect of retinoic acid on the number of cells stained with an antibody at twelve levels of intensity. The results are expressed as cumulative percentages. Channel 256 counts the most fluorescent cells and channel 1 the least. For example the band 121-256 represents all the cells counted in channels 121-256. This is referred to as high intensity in the text.

In calculating the percentage of cells stained, the number of autofluorescent cells was subtracted using the formula described in the text, (materials and method section of Chapter 3). There was no further increase in the number of cells in band 1-20, therefore for the sake of clarity this is not shown.

(MCA 45 = MRC OX-3

(MCA 64 = 5041-16.3

Fig. 27: The effect of 10^{-6}M retinoic acid on H-2(KD) expression in F1 cells. The difference is statistically significant.

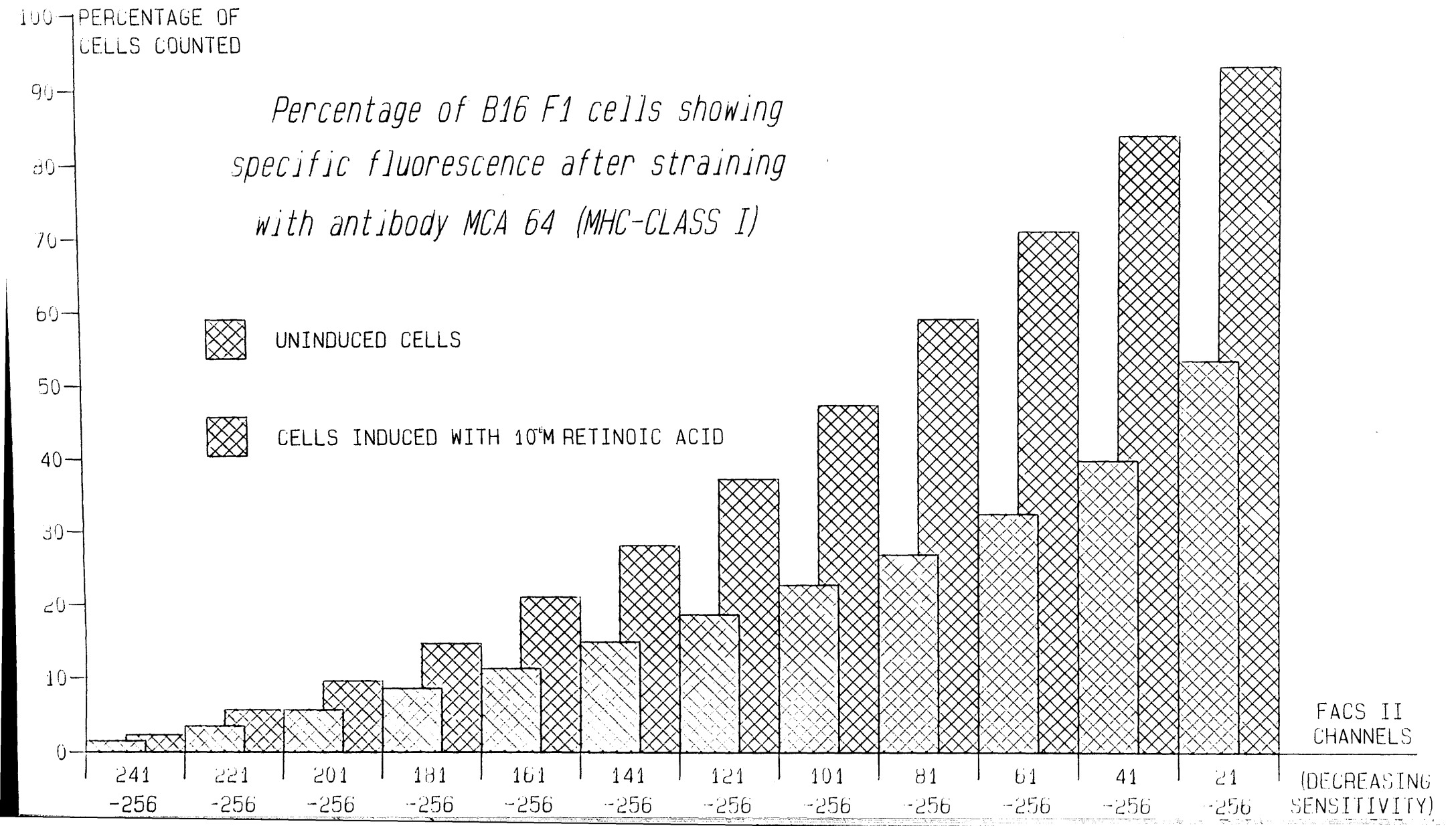

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F1 cells showing specific fluorescence after straining with antibody MCA 64 (MHC-CLASS I)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁶M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
61 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 28

This graph shows the effect of 10^{-6} retinoic acid on I-A expression in F1 cells. The increase is statistically significant.

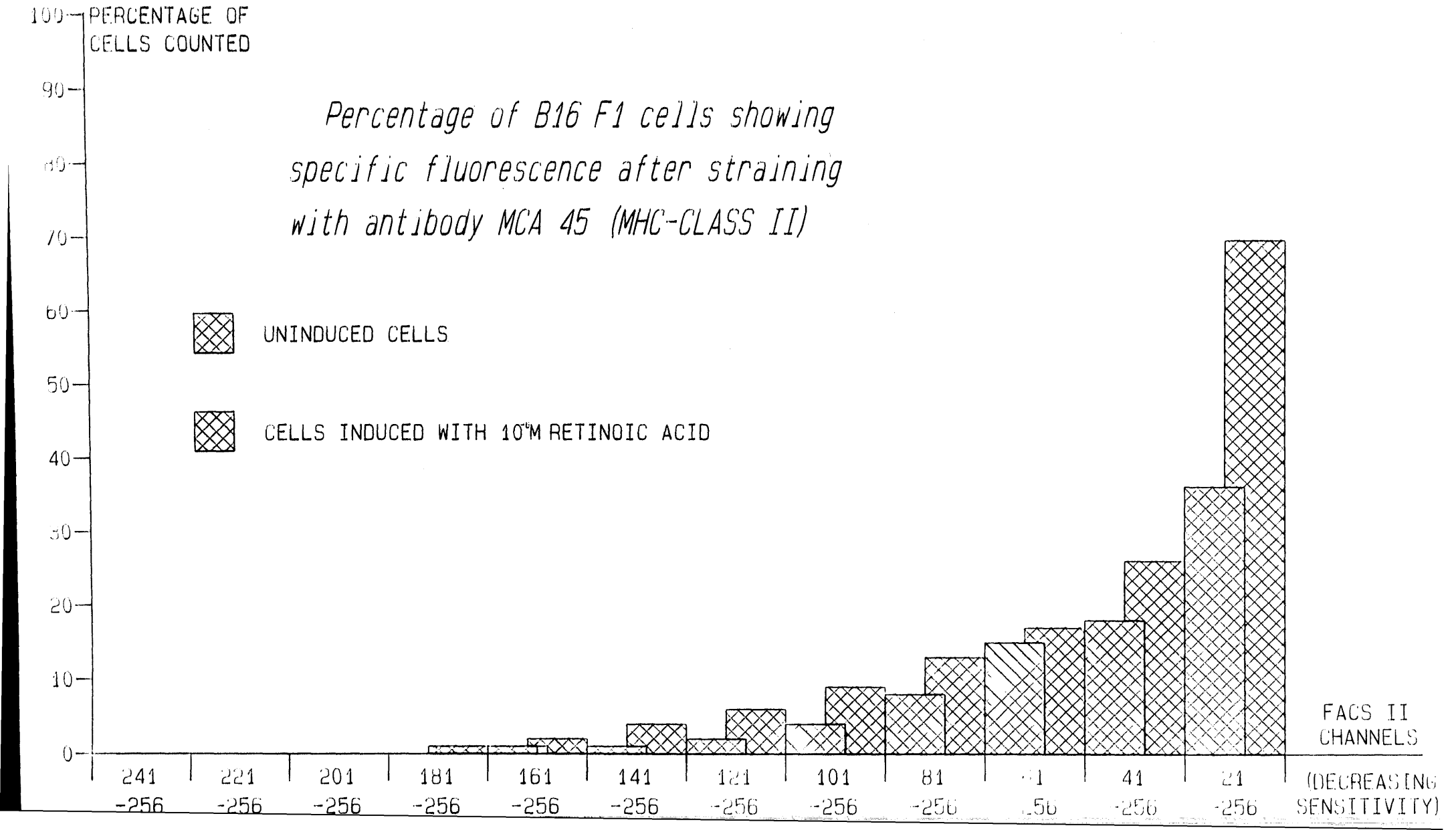

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F1 cells showing specific fluorescence after straining with antibody MCA 45 (MHC-CLASS II)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁶M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 29

This graph shows the effect of 10^{-6}M retinoic acid on H-2(KD) expression in F10 cells. The increase is statistically significant.

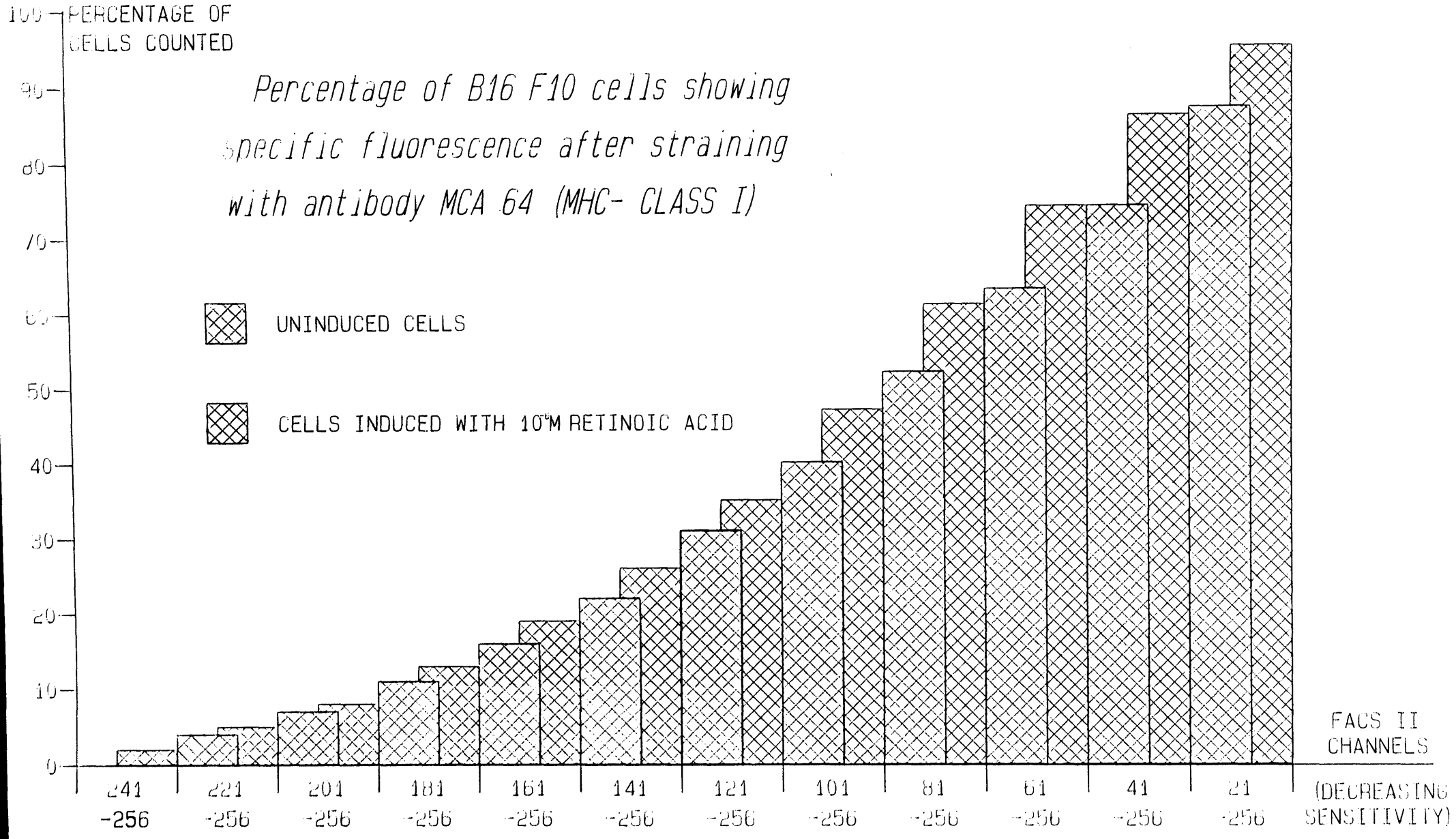

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F10 cells showing specific fluorescence after straining with antibody MCA 64 (MHC- CLASS I)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁶M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
61 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 30

This graph shows the effect of 10^{-6}M retinoic acid I-A expression in F10 cells. This increase is statistically significant.

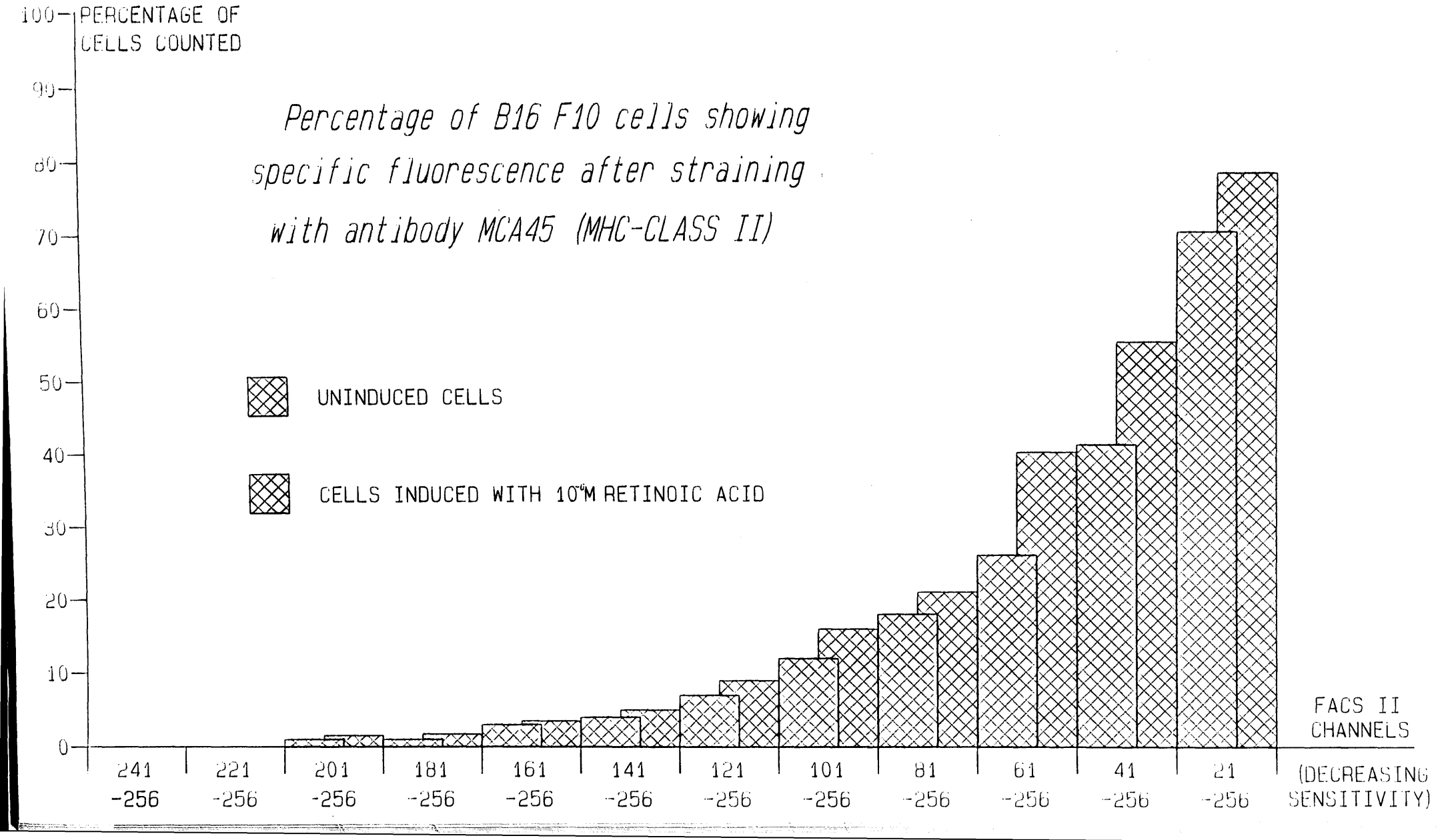

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F10 cells showing specific fluorescence after straining with antibody MCA45 (MHC-CLASS II)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁶M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
61 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 31

This graph shows the effects of 10^{-9}M retinoic acid on T-A expression in F1 cells. The difference is not significant.

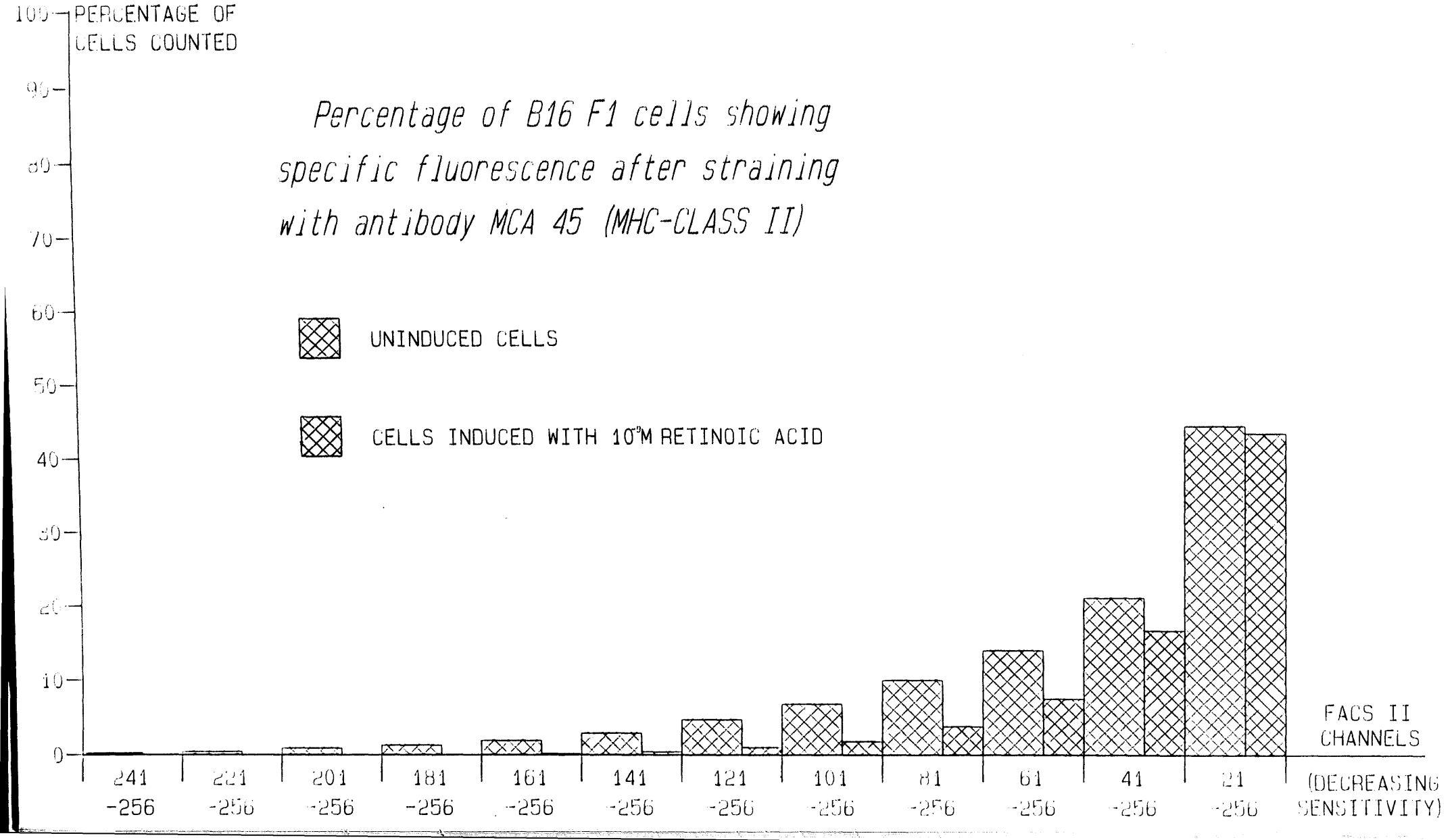

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F1 cells showing specific fluorescence after straining with antibody MCA 45 (MHC-CLASS II)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁹M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
61 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 32

This graph shows the effect of 10^{-9}M retinoic acid on F10 cells stained for H-2KD. The difference is not significant.

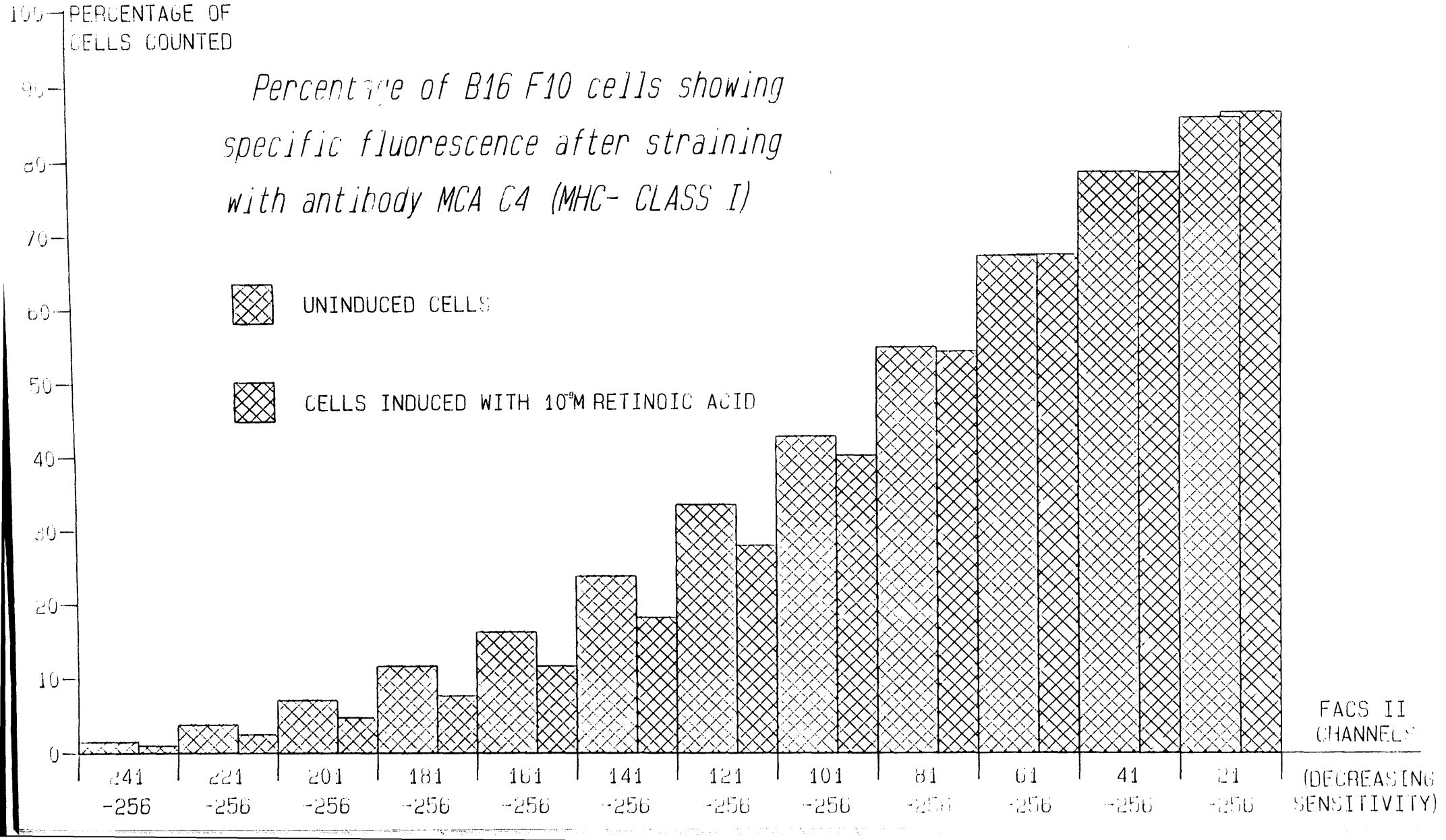

PERCENTAGE OF CELLS COUNTED
100
90
80
70
60
50
40
30
20
10
0
Percentage of B16 F10 cells showing specific fluorescence after straining with antibody MCA C4 (MHC- CLASS I)
UNINDUCED CELLS
CELLS INDUCED WITH 10⁻⁹M RETINOIC ACID
241 -256
221 -256
201 -256
181 -256
161 -256
141 -256
121 -256
101 -256
81 -256
61 -256
41 -256
21 -256
FACS II CHANNELS
(DECREASING SENSITIVITY)

FIGURE 33

The effect of retinoic acid on tumour forming ability of B16 F10 cells following IV injection. No tumours were seen with cells treated with 10^{-6}M retinoic acid. A significant decrease was seen with cells treated with 10^{-9} retinoic acid ($p < 0.0001$).

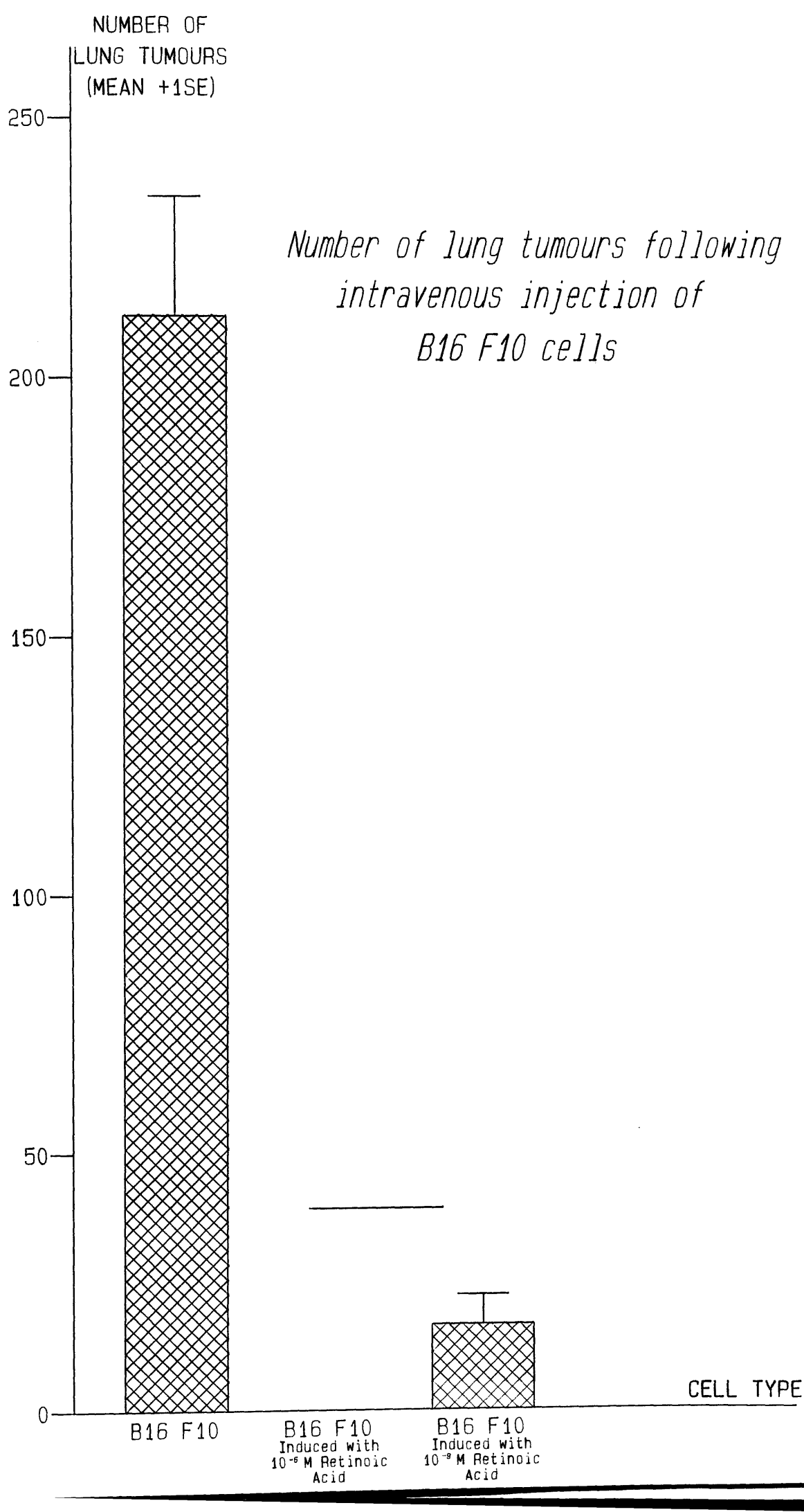

NUMBER OF
LUNG TUMOURS
(MEAN +1SE)
250
200
150
100
50
0
Number of lung tumours following
intravenous injection of
B16 F10 cells
CELL TYPE
B16 F10
B16 F10
Induced with
10⁻⁶ M Retinoic
Acid
B16 F10
Induced with
10⁻⁹ M Retinoic
Acid

FIGURE 34

The effect of retinoic acid on lung tumour formation by F1 cells. No tumours were seen with cells treated with 10^{-6}M retinoic acid. A decrease was seen with 10^{-9}M retinoic acid but was not significant.

Note: Change in y-axis scale from Fig. 33.

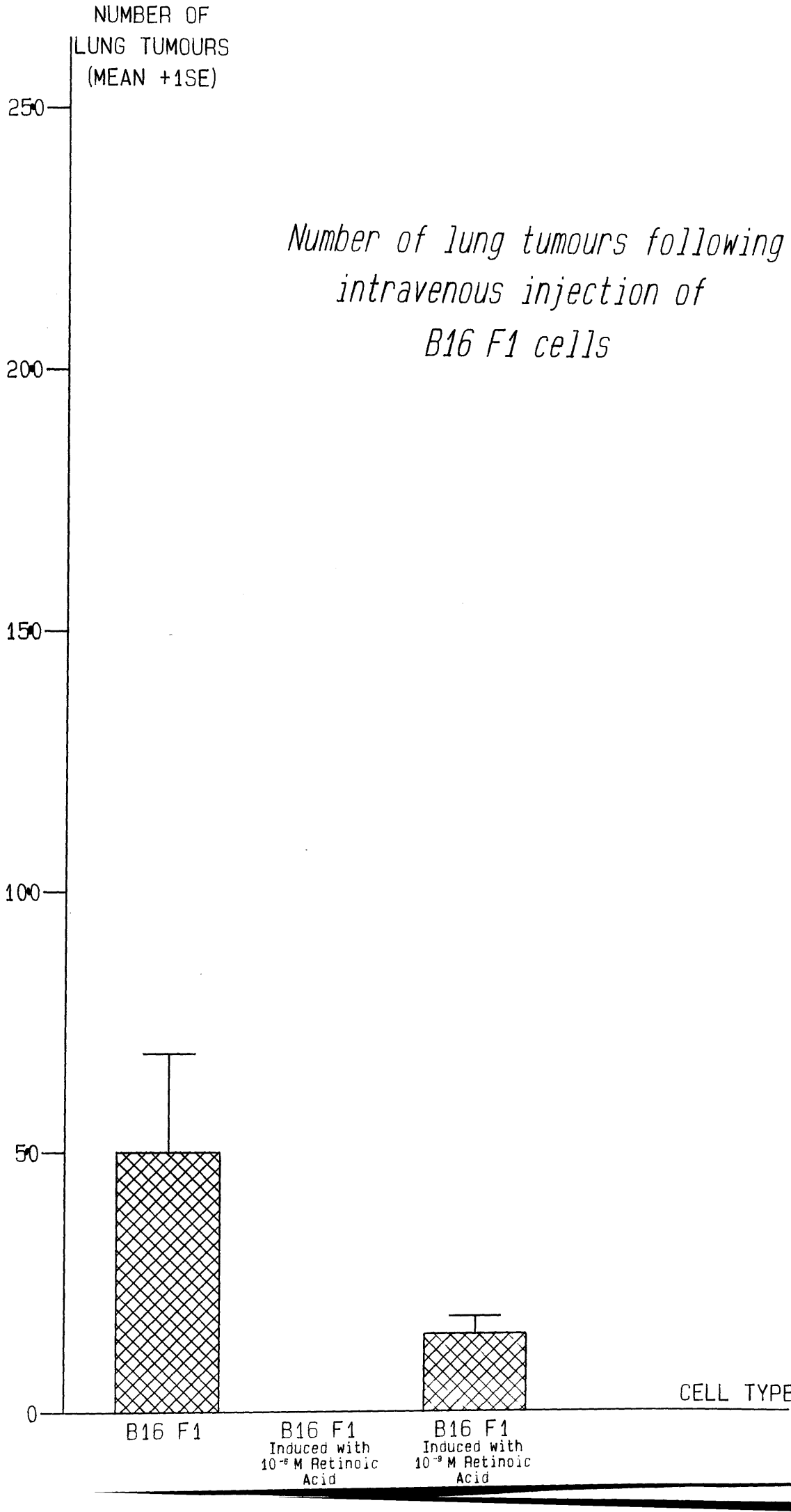

NUMBER OF
LUNG TUMOURS
(MEAN +1SE)
250
200
150
100
50
0
Number of lung tumours following intravenous injection of B16 F1 cells
CELL TYPE
B16 F1
B16 F1
Induced with
10⁻⁶ M Retinoic
Acid
B16 F1
Induced with
10⁻⁹ M Retinoic
Acid

FIGURE 35

The effect of retinoic acid on the formation of mesenteric tumours by F10 cells following IP injection. Cells induced with 10^{-6}M retinoic acid formed no tumour. A significant decrease was found at 10^{-9}M retinoic acid.

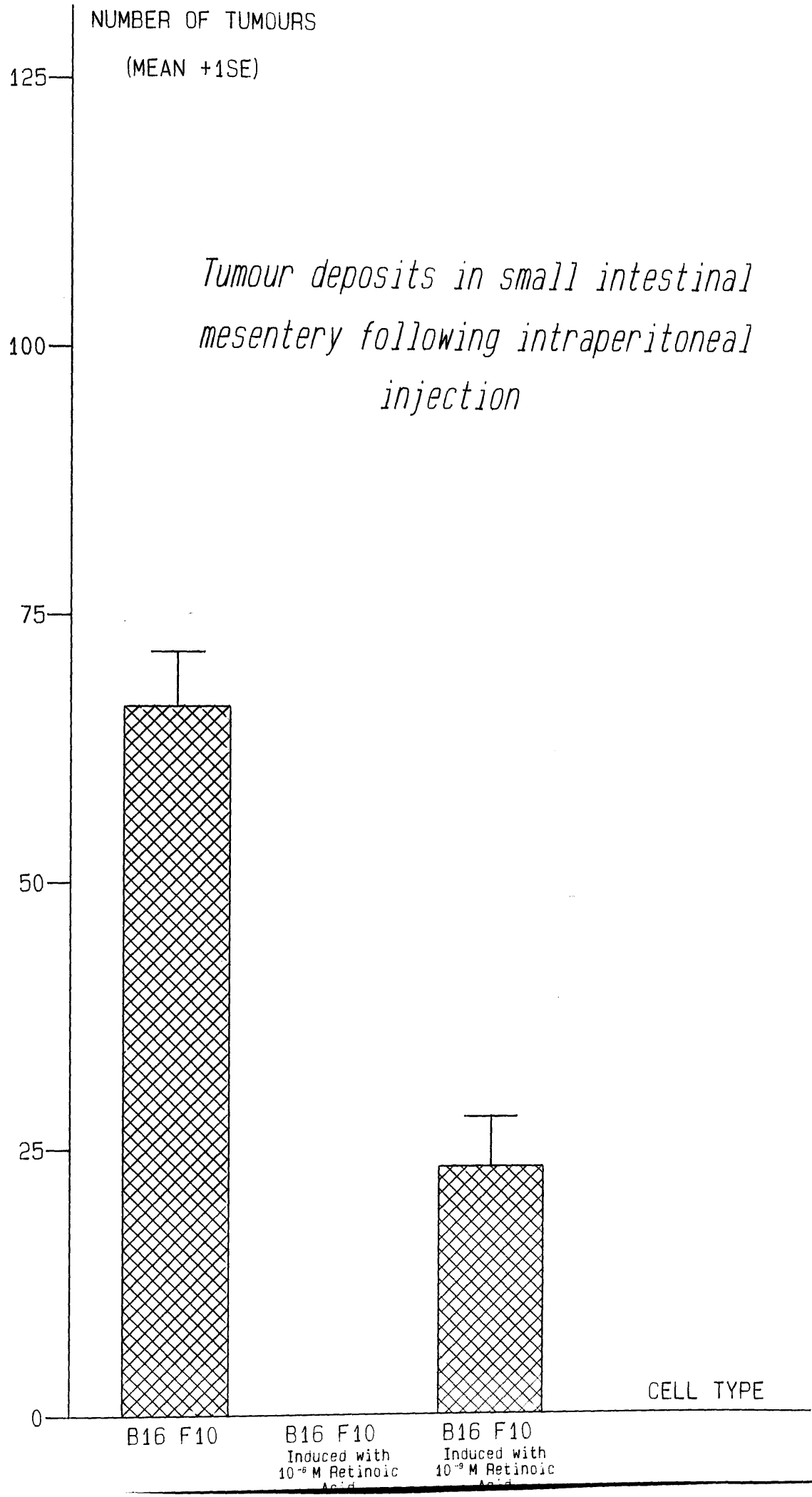

NUMBER OF TUMOURS
(MEAN +1SE)
125
100
75
50
25
0
Tumour deposits in small intestinal mesentery following intraperitoneal injection
CELL TYPE
B16 F10
B16 F10
Induced with
10⁻⁶ M Retinoic
Acid
B16 F10
Induced with
10⁻⁹ M Retinoic
Acid

FIGURE 36

The effect of retinoic acid on the formation of mesenteric tumours by F1 cells injected IP. No tumours were found with cells induced by 10^{-6}M retinoic acid. A significant decrease was seen at 10^{-9}M retinoic acid.

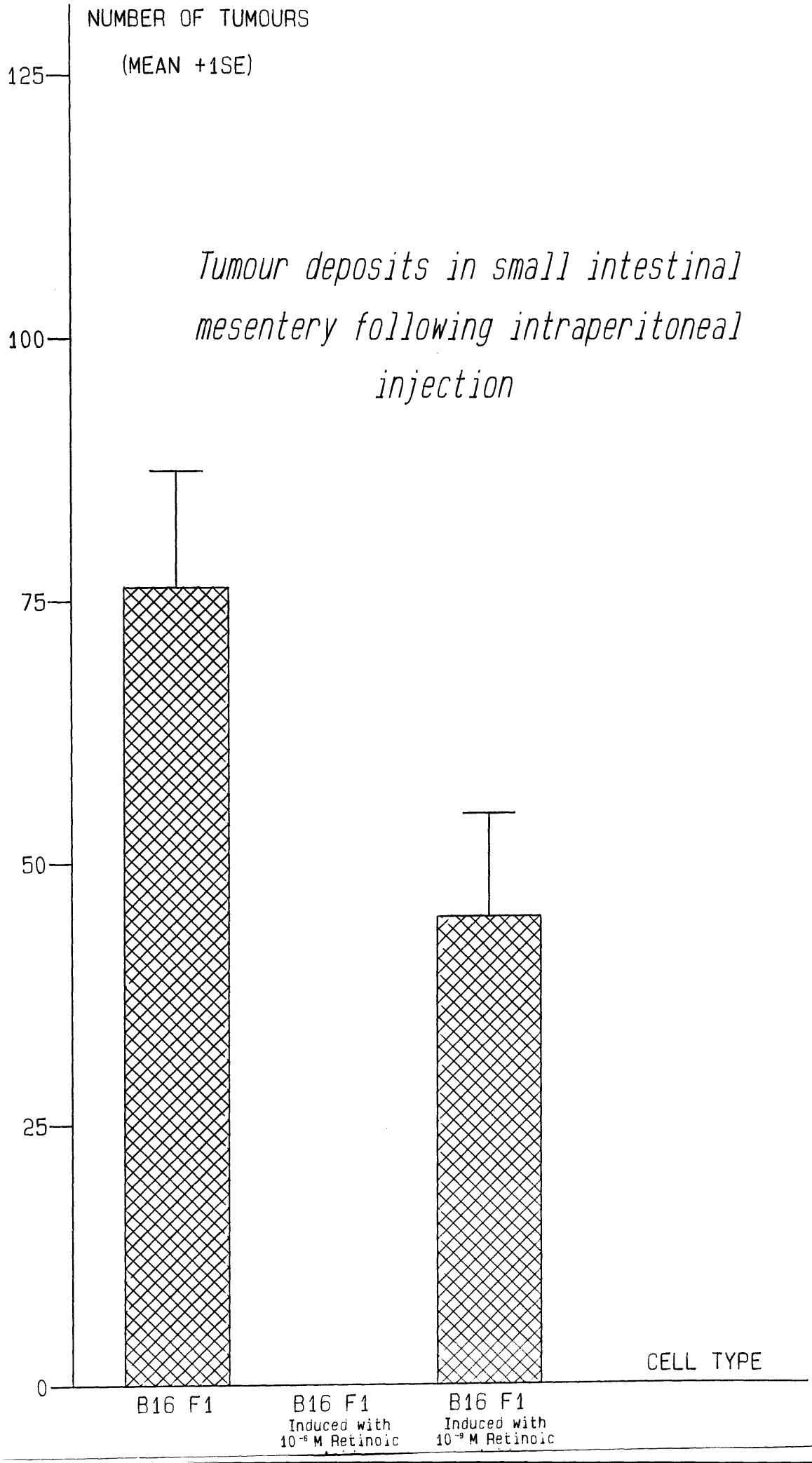

NUMBER OF TUMOURS
(MEAN +1SE)
125
100
75
50
25
0
Tumour deposits in small intestinal mesentery following intraperitoneal injection
CELL TYPE
B16 F1
B16 F1
Induced with
10⁻⁶ M Retinoic
B16 F1
Induced with
10⁻⁹ M Retinoic

FIGURE 37

The effect of retinoic acid on the trapping of radiolabelled F10 cells in the lung. A significant decrease is seen when cells were induced with 10^{-6}M retinoic acid. Cells induced with 10^{-9}M retinoic acid showed no significant difference.

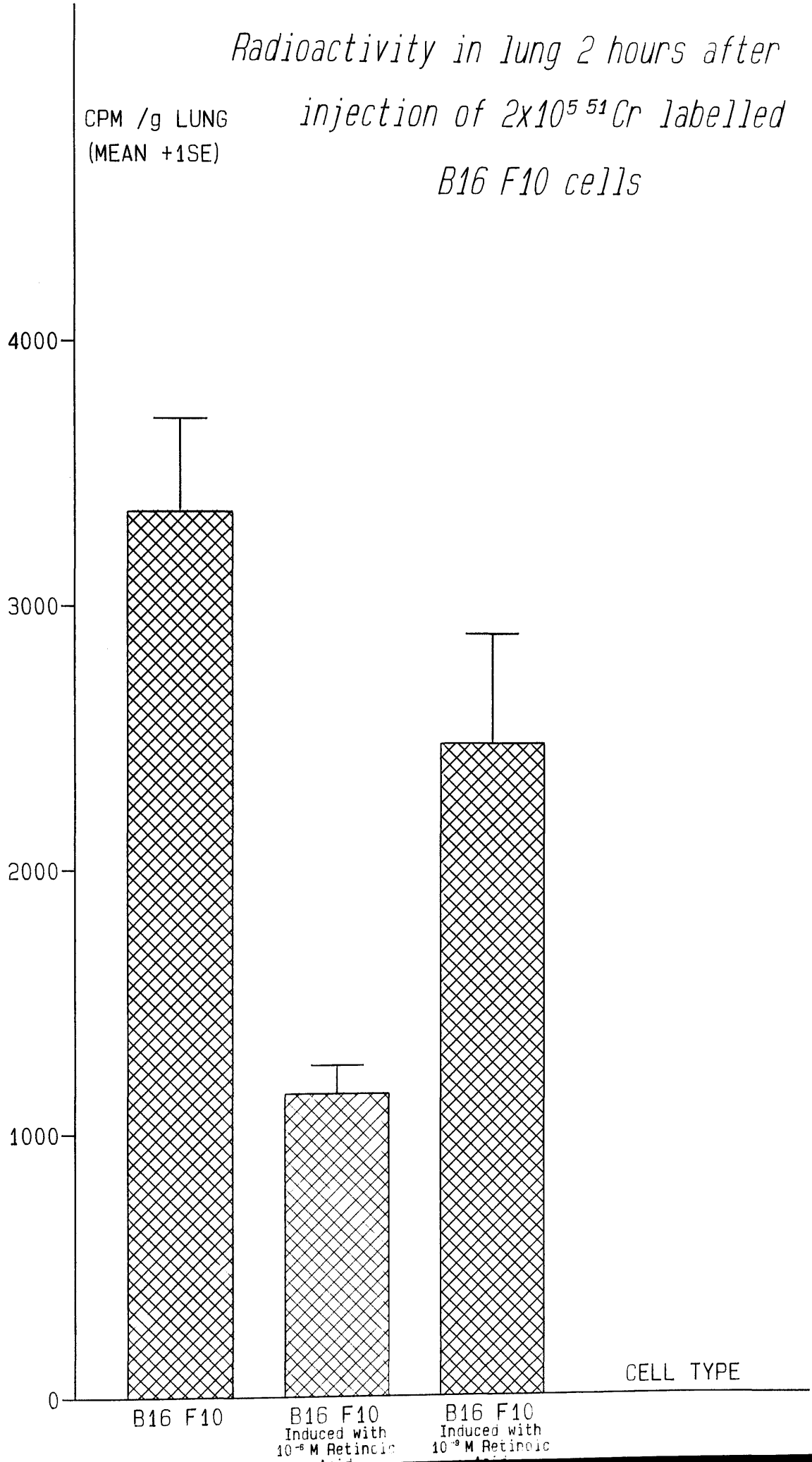

Radioactivity in lung 2 hours after injection of 2x10⁵ ⁵¹Cr labelled B16 F10 cells
CPM /g LUNG
(MEAN +1SE)
4000
3000
2000
1000
0
B16 F10
B16 F10
Induced with
10⁻⁶ M Retinoic
B16 F10
Induced with
10⁻⁹ M Retinoic
CELL TYPE

FIGURE 38

The effect of retinoic acid on trapping of F1 cells in the lung. A significant increase in cell trapping is seen when cells were induced with 10^{-6}M retinoic acid. No difference is seen when 10^{-9}M retinoic acid was used.

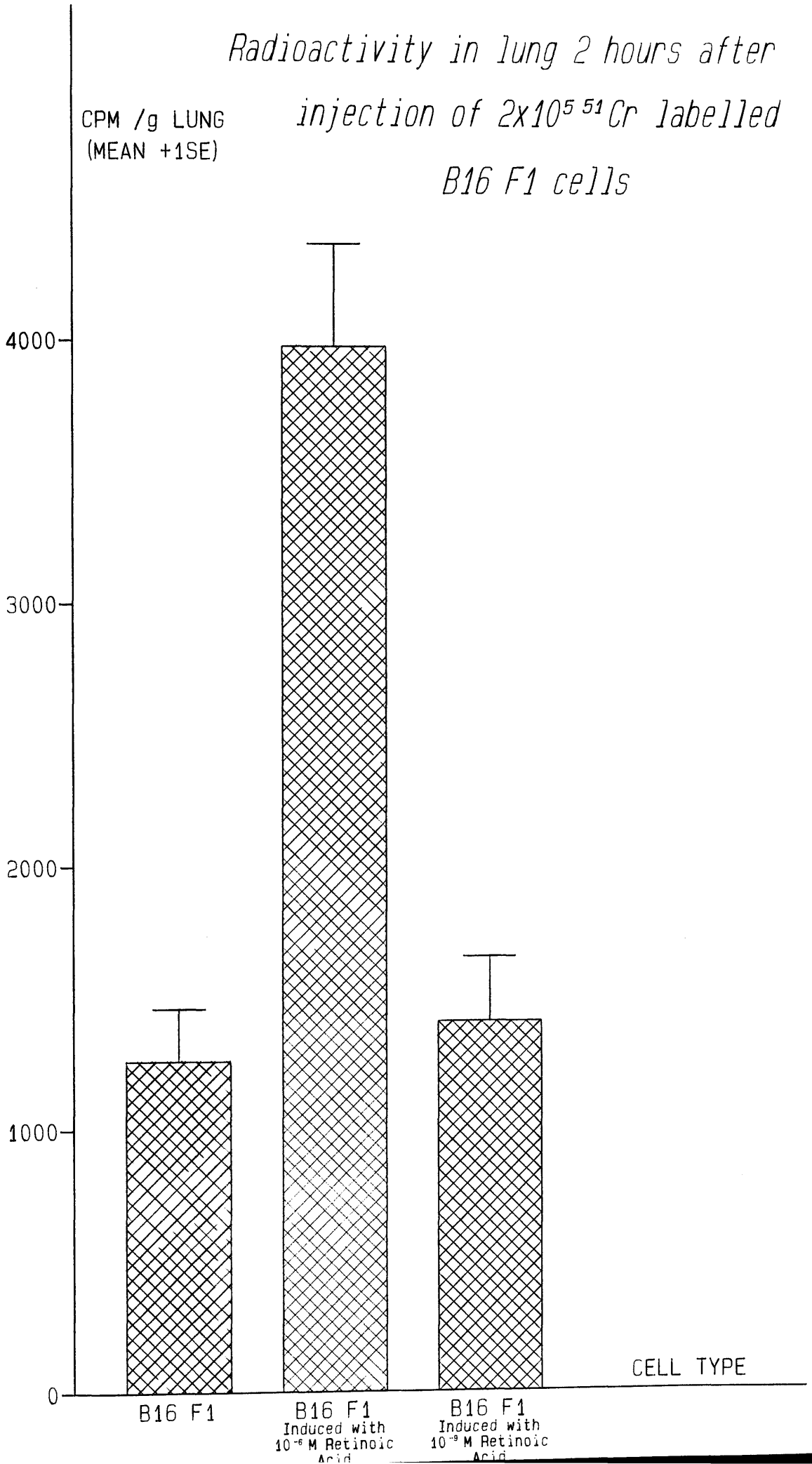
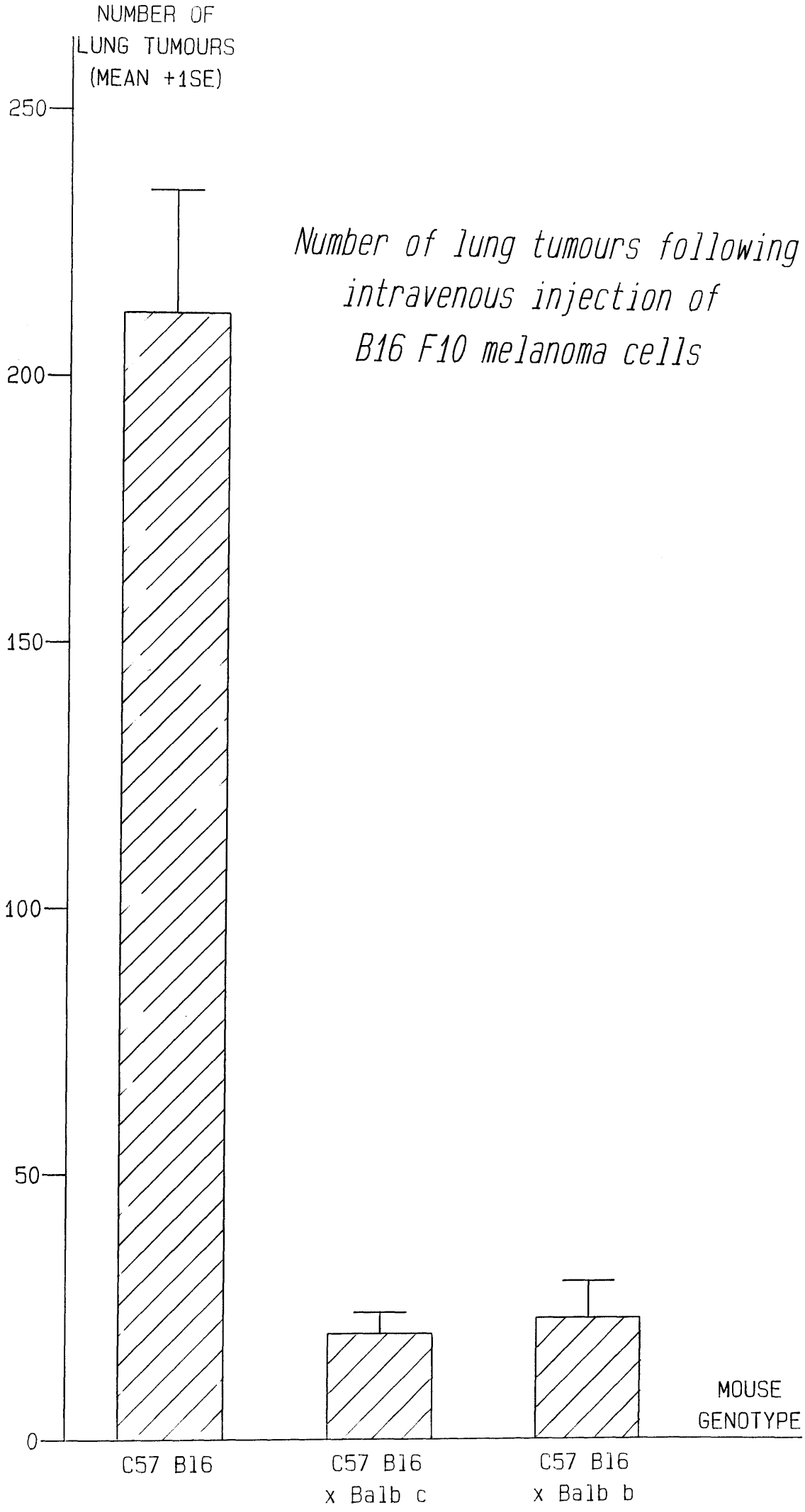

Radioactivity in lung 2 hours after injection of 2x10⁵ ⁵¹Cr labelled B16 F1 cells
CPM /g LUNG
(MEAN +1SE)
4000
3000
2000
1000
0
CELL TYPE
B16 F1
B16 F1
Induced with
10⁻⁶ M Retinoic
Acid
B16 F1
Induced with
10⁻⁹ M Retinoic
Acid

FIGURE 39

Injection of F10 cells into hybrid animals which were with heterozygous (b x k) or homozygous (b x b) at H-2 resulted in a significant decrease in the number of lung tumours as compared to the syngeneic strain (C57 B16).



Number of lung tumours following intravenous injection of B16 F10 melanoma cells

FIGURE 40

Comparison of the ability of F10 cells to form mesenteric deposits in the syngeneic strain (C57 B16) and hybrid strain (C57 B16 x balb c). This difference is not significant.

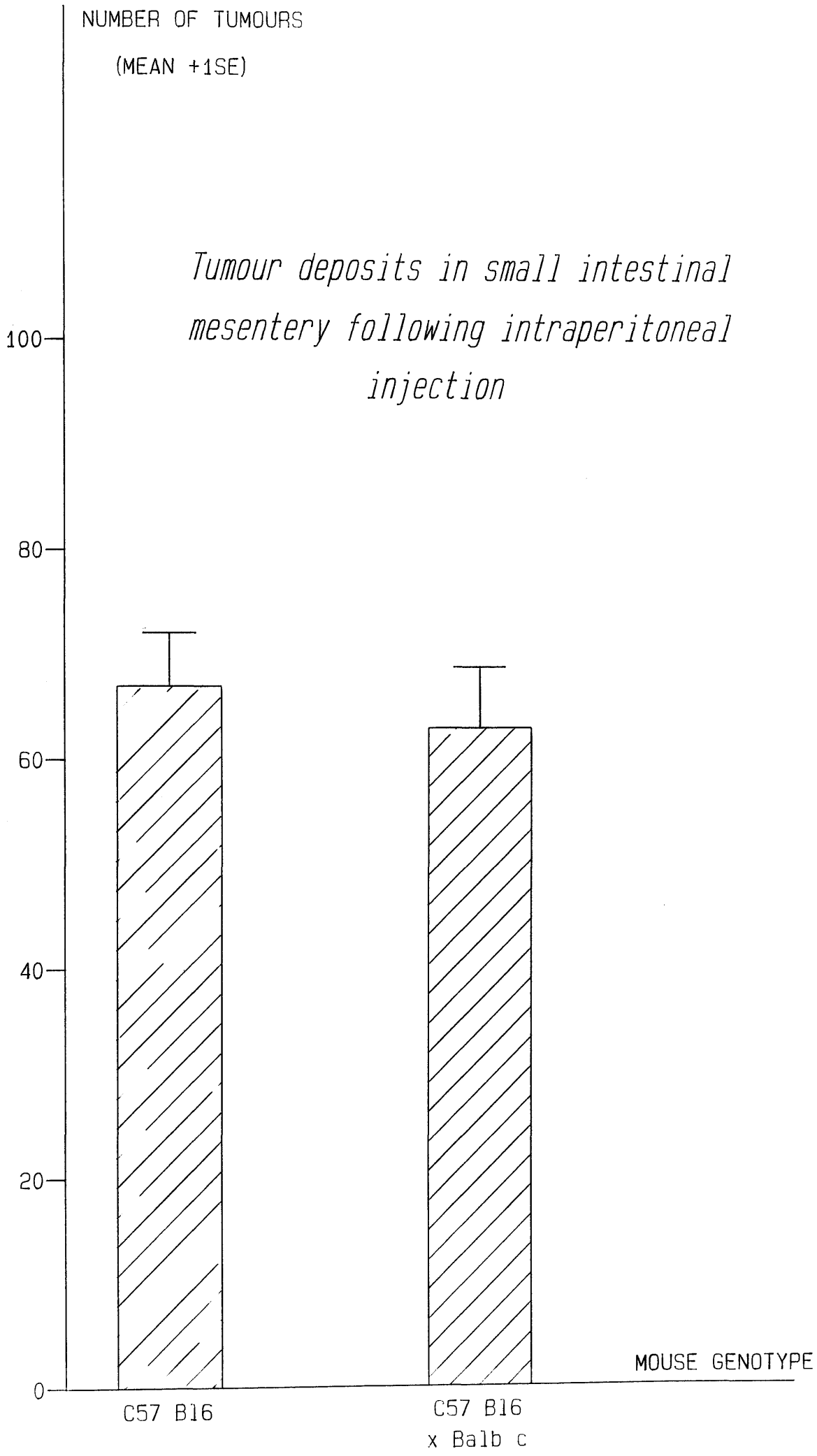

NUMBER OF TUMOURS
(MEAN +1SE)
Tumour deposits in small intestinal mesentery following intraperitoneal injection
100
80
60
40
20
0
MOUSE GENOTYPE
C57 B16
C57 B16
x Balb c

FIGURE 41

Comparison of lung trapping of radiolabelled F10 cells in syngeneic (C57 B16) and hybrid (C57 B16 x balb c) animals measured at 2 hours. There is no significant difference.

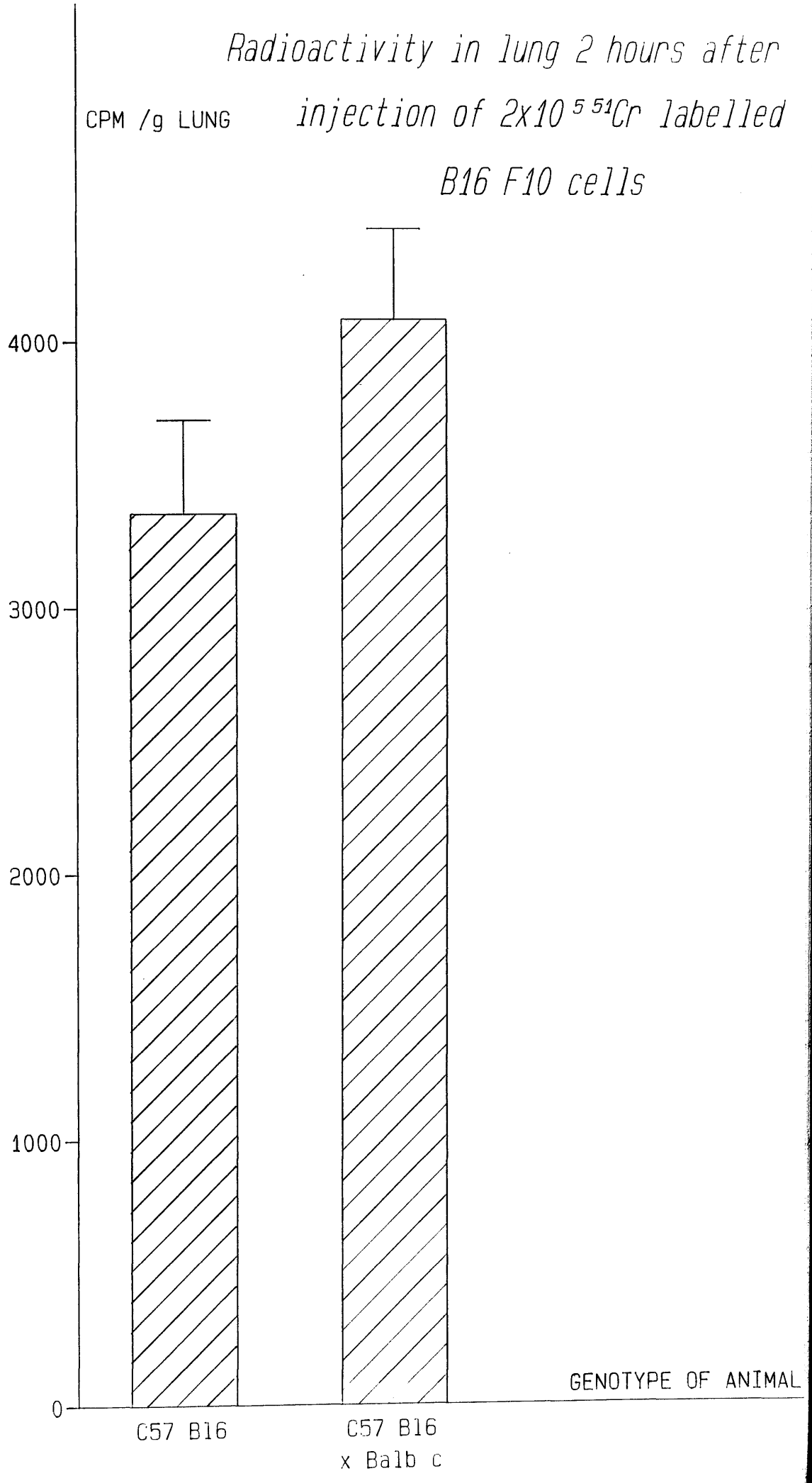
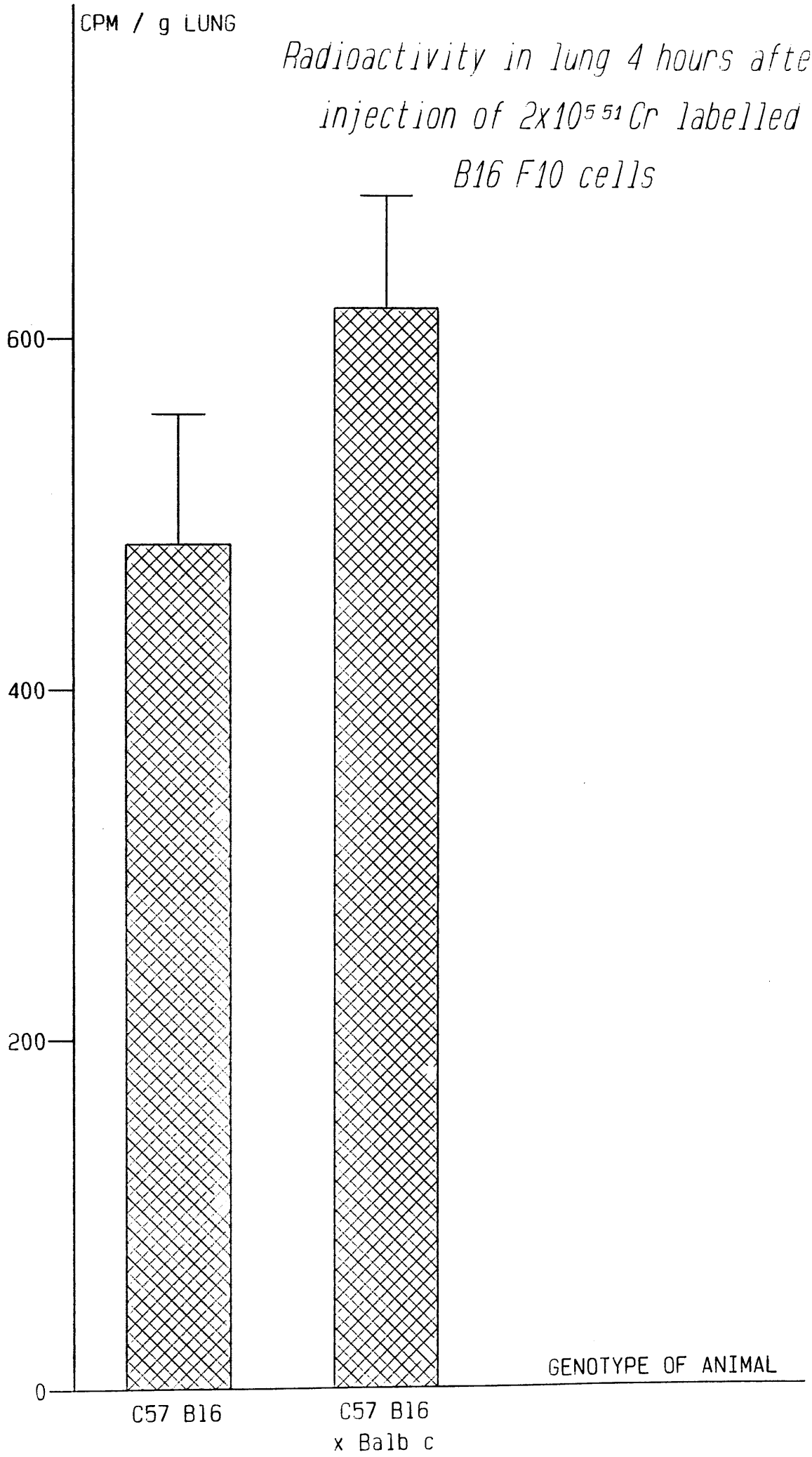

Radioactivity in lung 2 hours after injection of 2x10 5 51Cr labelled B16 F10 cells
CPM /g LUNG
4000
3000
2000
1000
0
C57 B16
C57 B16 x Balb c
GENOTYPE OF ANIMAL

FIGURE 42

Comparison of lung trapping of F10 cells in syngeneic and hybrid animals at 4 hours. The difference is not significant. Note the decrease in total radioactivity as compared with Fig. 41.


CPM / g LUNG
Radioactivity in lung 4 hours after injection of 2x10⁵ ⁵¹Cr labelled B16 F10 cells
600
400
200
0
GENOTYPE OF ANIMAL
C57 B16
C57 B16 x Balb c

APPENDIX 1

Solutions used in Tissue Culture

Medium A

1. Nutrient Mixture Ham F10 (Tenfold concentrate)	20ml
2. 7.5% solution of $NaHCO_3$	1ml
3. GPSA solution (200mM c-Glutamine) (5000 iu/ml Penicillin) (5000 iu/ml Streptomycin) (250 µg/ml Amphotericin B)	5ml
4. 2.95% Tryptose Phosphate in water	20ml
5. Foetal Calf Serum	20ml
6. 20mM Hepes in water.	180ml

Medium B

1. Medium A without Foetal Calf Serum 13mls	
2. Foetal Calf Serum	6ml
3. Glycerol	1ml

Hepes Buffered Saline

1. Na Cl	8g)	
2. K Cl	0.4g)	made up to 1 litre in deionised
3. Glucose	1g)	water.
4. Hepes	2.4g)	

APPENDIX 2

Radiolabelling of B16 cells and measurement of cell trapping

A. Labelling

B16 cells in medium A.

↓

Replace with medium A containing 3MBq/100ml of $Na^{51}Cr$

↓

Incubate overnight

↓

Wash x 2 in MEM and resuspend to 10^6 cells ml^{-1}

↓

radioactivity in 0.1ml aliquot of cells measured.

B. Trapping

2×10^5 labelled cells injected IV.

↓

2 or 4 hours later - lungs removed and weighed.

↓

Radioactivity counted - expressed as cpm/g (wet weight)

C. Calculaton

$$\frac{\text{cpm/g} \times 1000}{\text{cpm in 0.1ml of cell suspension}} = \text{cpm/g corrected for efficiency of cell labelling.}$$

APPENDIX 3

The mouse H-2 major histocompatability complex

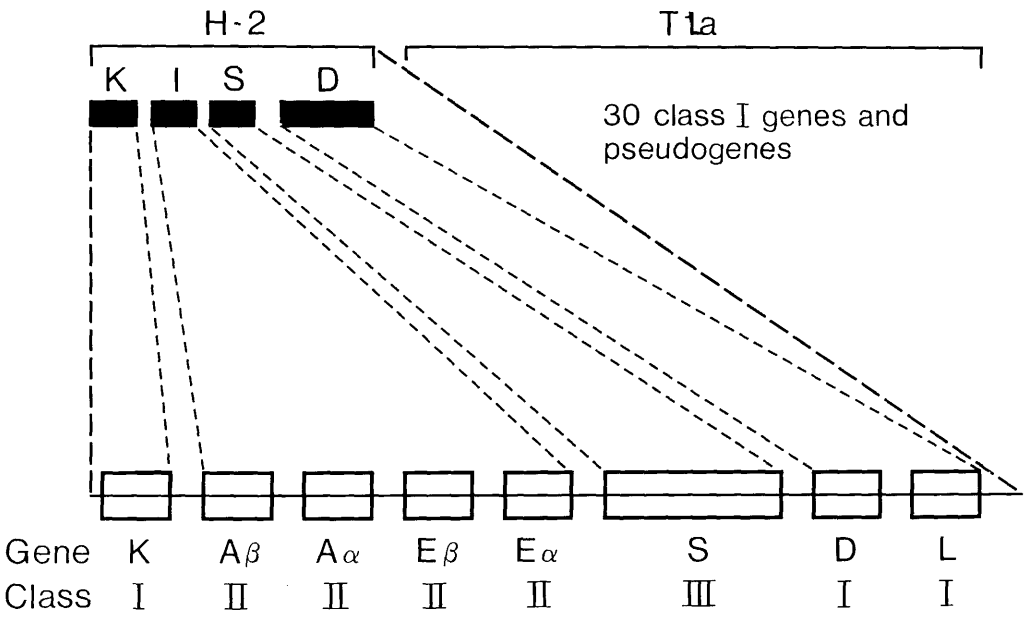
In the H-2 notation the superscript lower case letter eg H-2b denotes the haplotype. The haplotype is a unique combination of shared and unique antigenic determinants.

Each MHC class I gene product (K,D and L) has both individual and shared class I specificities.

e.g. gene product	Antigenic Determinants.
H-2Kd	3, 8, 22, 28, 29, 34, 46, 47, 31*.
H-2Dd	3, 6, 13, 27, 28, 29, 35, 36, 41, 42, 43, 44, 49, 4*.

* unique determinants.

Chromosome 17



APPENDIX 4

Mouse Histocompatability Genotypes

GENE	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-12	H-13	H-14
CHROMOSOME	7	17	2	7	*	2	*	*	*	*	2	*
STRAIN												
C57 B16	c	b	a	a	not a or b	a	a	a	not a or b	a	a	b
Balb c	b	d	c	*	*		b	c	b	a	d	*
Balb b	b	b	c	*	*	*	b	c	b	a	d	*
C57 BL10	c	b	a	a	*	a & b	a	a	a	a	a	b

* not known

not a or b - not precisely typed.

a & b - reacts with sera to a and b.

Data supplied by Olac Ltd, Blackthorn, Oxfordshire.

APPENDIX 5

Method for Natural Killer Cell Assay.

A. Target cell Preparation

Cultures of B16 F10 cells

↓

18 hours in Medium A with 3MBq/100ml of $Na^{51}Cr$

↓

Wash in Medium A.

↓

5 x 10^4 cells in 50µl of medium per well.

B. Effector Cells Preparation

Spleen from C57 B16 or C57 B16 x Balb c.

↓

Cut and compressed to remove lymphoid cells.

↓

Washed in medium A.

↓

1 hour at 37°C to remove adherent cells.

↓

Resuspend and add to microtitre wells in varying proportions.

C. Control

i) Target cells incubated with medium (S).

ii) Target cells incubated with Nonindet P-20 (M).

D. Results

$$\text{NK cytotoxicity} = \frac{T - S}{M - S} \times 100$$

Where T is radioactivity in wells with effector cells added.

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