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THE EFFECT OF IMMUNE STIMULATION AND IMMUNE SUPPRESSION
ON EXPERIMENTAL PRE-INVASIVE AND EARLY INVASIVE CARCINOMA
OF CERVIX.

A THESIS SUBMITTED BY ;

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DOCTOR OF MEDICINE

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DEPARTMENT OF PATHOLOGY

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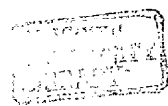
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SUMMARY

This thesis comprises a study of the pre-invasive and early invasive phases of a chemically induced carcinoma of uterine cervix, and the effects on the course of development of these of immune stimulation with Bacille Calmette-Guerin or immune suppression with anti-lymphocyte serum.

The aims of the experiments are to investigate the effects of immune stimulation or immunosuppression on :

- (1) the time from carcinogen implantation to the detection of the first malignant cells by cytology, and
- (2) the progression or regression of the various stages of pre-malignancy and early malignancy in the suggested progression :
normal → moderate dysplasia → severe dysplasia and / or carcinoma in-situ → carcinoma, with and without the continuing presence of the carcinogen.

Part I describes the experimental model and the reasons for its use. The model is a variation of that first described by Murphy in which, in mice, carcinoma of cervix is induced by inserting a thread, with a knot at one end impregnated with a carcinogen-beeswax mixture, through the cervical canal so that the impregnated knot rests against the cervix. The main reasons for using this model are : -

- (1) It gives a more reproducible incidence of tumours in the cervix than other methods, such as painting with carcinogen.

- (2) The development of the lesions can be followed and staged by the use of exfoliative cytology. This allows B.C.G. or A.L.S. to be given at a stage in the development when some definite pathological change in the progression to malignancy can be demonstrated. This avoids the necessity of giving the agents blindly, or the complications that might arise from the use of traumatic biopsy as a means of estimating the stage of development.
- (3) To reduce the complications due to the presence of the carcinogen, such as any immunosuppressive effect, the thread can be cut and the carcinogen removed. This also minimises any continuing carcinogenic effect such as might occur in short duration experiments in which large doses of carcinogen are given, with the result that the carcinogenic effect continues after the effect of immune stimulation has ceased. It also reduces the possibility of a massive carcinogenic effect overwhelming more subtle alterations in the immune status.

Part I also describes the experimental design which essentially consists of two groups of experiments, in the first of which the carcinogen remains in position throughout the experiment and in the second of which the carcinogen is removed as soon as the first cytologically malignant cells are detected. A.L.S. or B.C.G. are given when, in individual mice, a recognised stage in the development of carcinoma is reached.

Part II reviews the literature regarding A.L.S. emphasising the variable aspects of its effect on cellular and humoral immune responses to various antigens, and to different types of allografts, in differing strains and species of animals, where these have a bearing on tumour immunity. Also emphasised is the fact that many experiments have been performed without control groups using normal serum, compatible with the A.L.S., so that any toxic or antigenic stimulatory effect of the serum constituents may be missed.

Part III reviews the literature regarding B.C.G. and immune stimulation, and emphasises the numerous possibilities for varying immune responses to B.C.G. such as strain differences, preparation differences, and genetic drift in the bacteria, and variable human and animal responses in the recipients, and how these can affect the comparison and interpretation of experimental results.

Part IV gives details of the preliminary experiments. The first correlated the cytological findings with the histological appearances in the progression to carcinoma in the experimental animals and demonstrated that within the limits of the experiment the cytological findings accurately reflected the histologic appearances. The second demonstrated that the tumours produced in the cervix by this technique were immunogenic and demonstrated a difference in the antigenic strength of the tumours produced by the two methods.

Part V gives the results obtained which can be summarised as :

- (1) There is no alteration in the length of time from the implantation of the carcinogen to the detection of the first cytologically malignant cells in any groups.
- (2) The length of time from implantation to invasion is little altered in the experimental groups where the carcinogen remains in position, but that where the carcinogen is removed B.C.G. increases and A.L.S. decreases the pre-invasive stage.
- (3) Where the carcinogen remains in position the epithelium does not revert to normal in any group but remains cytologically malignant.
- (4) A.L.S. increases and B.C.G. decreases the number of tumours resulting from the pre-invasive stages where the influence of the carcinogen is removed.
- (5) B.C.G. will arrest the progression of some early invasive lesions, while from this stage, A.L.S. increases the resulting number of tumours, in those experiments where the carcinogen has been removed.

Part VI discusses the results in relation to other experiments.

INTRODUCTION

Although the specific immunising potency of methylcholanthrene induced tumours of mice had been suggested earlier ⁽¹⁾, the first convincing demonstration of immunity to methylcholanthrene induced tumours in inbred mice, was the destruction of the tumours following ligation of their blood supply ⁽²⁾. Shortly after comparable findings were demonstrated with methylcholanthrene induced rat sarcomas ⁽³⁾. The immune specificity to the transplanted sarcoma neoantigens was demonstrated by the finding that tumour immunised mice could still accept skin grafts from the tumour donor thus removing the possibility of the involvement of an alloantigenic immune response ⁽⁴⁾, and the demonstration that resistance to tumour re-implantation could develop in the autochthonous host excluded the possibility that the immunity could be accounted for by residual heterozygosis ⁽⁵⁾.

Since then a large number of chemically induced tumours have been shown to be antigenic ⁽⁶⁾ and many of these tumours have been used to analyse the involvement of tumour-host interactions in the process of oncogenesis, and to demonstrate the effects of host immune stimulation ^(7,8) or immunosuppression ^(9,10) on subsequent tumour growth.

Little has been reported on the effect of manipulation of the immune response on identifiable pre-invasive stages of carcinoma development ^(11,12,13), although in some studies immune stimulation or immunosuppression has been started blindly at times when malignant clones of cells might be expected to be present, but before outgrowth of these has occurred ⁽¹⁴⁾.

Clinically large numbers of lesions thought to precede invasive carcinoma of cervix have been detected by the use of exfoliative cytology, and it has been concluded that only about 25 - 30 % of these pre-invasive lesions will progress to an invasive phase ^(15,16). Evidence has also been produced that pre-cancerous lesions of the bronchi disappear, in ex-smokers, within five years of giving up smoking ⁽¹⁷⁾.

Whether immunological factors are involved in the development and progression or regression of these lesions is unknown, but the high incidence of pre-invasive cervical lesions found in renal transplant patients undergoing immunosuppressive therapy ⁽¹⁸⁾ and the suggested immunodepressive effect of cigarette smoking ⁽¹⁹⁾, suggests that they may be a factor. Premalignant and malignant skin tumours also appear to be greatly increased in immunosuppressed patients ⁽²⁰⁾ and experimentally there is some evidence that very early chemically induced premalignancies of liver may often be suppressed immunologically ⁽¹³⁾.

The object of the experiments described in this thesis is to consider any modification of the development, progression or regression of cytologically demonstrated pre-invasive or early invasive cervical lesions, by non-specific immune stimulation using Bacille Calmette-Guerin (B.C.G.) or immunosuppression using anti-lymphocyte serum (A.L.S.).

Part I

EXPERIMENTAL MODEL AND REASONS FOR ITS USE

After the demonstration of the carcinogenic properties of crude tar⁽²¹⁾ attempts were made to induce carcinoma of cervix in mice by the instillation or implantation of crude tar into the vaginal vault or cervix^(22,23). Only a few invasive carcinomas resulted from these experiments the lesions often regressing despite continued application of the tar.

Experimental induction of malignant tumours by the use of crude tar was eventually replaced by more refined methods utilising pure compounds possessing potent carcinogenic properties which resulted in higher yields of epidermoid carcinomas. The intravaginal instillation of a 1% solution of 3, 4 benzpyrene in acetone induced a high incidence of carcinoma of cervix in C₃H mice⁽²⁴⁾, and painting the cervix, under direct vision through a speculum, resulted in an even greater yield of tumours with a shorter latent period⁽²⁵⁾.

Accurate sustained placement of the carcinogen in the uterine cervix resulting in a more constant incidence of carcinoma was attained by inserting a thread saturated in methylcholanthrene into the cervical canal under direct vision⁽²⁶⁾, and this technique has been used subsequently to study the development of experimental carcinoma⁽²⁷⁾ and pre-cancerous lesions^(28, 29) of cervix, by histology and exfoliative cytology. We have adapted this model and used it in this study of the early lesions of experimental chemical carcinogenesis, and the effects thereon of non-specific immunostimulation and immunosuppression.

One advantage of using this technique is that the thread containing the carcinogen can be removed at any time thus reducing or eliminating any possible immunosuppressive effect of the carcinogen and allowing the development of the tumour without this factor complicating the experimental immunological manipulations. This is of importance since it not only reduces the risk of systemic immunosuppression, which can be detected and monitored, but it also reduces the possibility of local immunosuppressive activity, which may be of importance in the development of neoplasia and the effectiveness of any immune response to the tumour.

Using the uterine cervix as the organ for the induction of carcinoma by chemical agents also gives an advantage over other sites in that, with the use of exfoliative cytology, the early changes can be followed and studied by a non-traumatic, non-destructive process, in contrast to sequential biopsies which might, by removal of tissue, affect subsequent tumour development, or by causing an inflammatory reaction, alter the immune response to the tumour.

Using exfoliative cytology the sequence of changes, normal → dysplasia → severe dysplasia and/or carcinoma in-situ → invasive carcinoma, can be readily identified and followed; the onset and duration of these phases can be timed in relation to the date of implantation of the carcinogen, and neoplastic tumour formation can also be differentiated from inflammatory swelling with which it could be confused if only palpation is used to determine tumour occurrence.

Removal of the carcinogen also has the advantage of producing a long latent period and slow growing tumours with a low growth fraction which approximate more closely to human tumours than most experimental models, e.g. the subcutaneous injection of chemical carcinogens in animals tend to produce fast growing sarcomas which may have little relevance to most human tumours ⁽³⁰⁾.

Another advantage of this system is that the tumour induced is a carcinoma, occurring at a site which is a major problem in humans, and preceding which, in the cervical epithelium, all the pre-invasive changes can be demonstrated which if associated with cellular antigenic changes ⁽³¹⁾ might condition the host's subsequent immune response to the tumour, in contrast to the injection of tumour cells into immunologically virgin animals.

The use of primary tumour in outbred hosts may also approximate more closely to the variability and unpredictability characteristic of human neoplasms and emphasises those features of immune surveillance, neoplastic progression and spread which may be unique to undisturbed in-situ tumours, in contrast to transplants of cultured tumour cells, or subcutaneous induced sarcomas. It has been suggested from the results of experiments using pellets of 3 - methylcholanthrene in wax, implanted into the skin, that tumours induced by low concentrations of oncogen may be good models of spontaneous neoplasms ⁽³²⁾ and this strengthens the hypothesis that some spontaneous tumours may develop from exposure to low levels of oncogen. In some of our experiments the attempt to reduce the total exposure to the carcinogen was made by removal of the carcinogen in the early stages of the neoplastic process, so that the exposure to the carcinogen is for a relatively short duration.

Materials and Methods

Animals Female virgin Swiss mice aged 6 - 9 weeks at the beginning of the experiment, and weighing about 30 gms were used. The mice were housed 5 per cage and given drinking water and purina chow ad libitum, and housed in temperature and light controlled rooms.

Carcinogenesis The carcinogen was 20 - Methylcholanthrene (Koch Light Laboratories) suspended in beeswax in a ration 20 MC : Beeswax of 1 : 3.

The carcinogen was applied using a slight modification of a technique first described by Murphy ⁽²⁶⁾. Sterile No. 10 cotton threads, knotted by a triple loop, 1 cm from one end, were impregnated with the carcinogen-beeswax mixture by dipping the knot into the melted mixture using forceps. The lengths of thread on either side of the knot were protected from contamination with the mixture by the blades of the carrying forceps. The threads were weighed before and after impregnation and only threads carrying \pm 0.25 mg carcinogen were used.

Blunt pointed tapestry needles were used to carry the thread.

Using ether anaesthesia, deep enough to obtain complete relaxation, with the dorsum of the left hand against the back of the mouse and the tail held between the left thumb and forefinger, pulling the tail sufficiently to arch the back over the dorsum of the hand, exposed the vagina and allowed the insertion of the tip of the needle. With sufficiently deep anaesthesia the needle could readily be inserted into the cervix without trauma, passed through the lateral horn of the uterus and out through a small incision in the

left flank. The end of the thread was anchored in the subcutaneous tissues so that the carcinogen impregnated knot was maintained in contact with the uterine cervix. The skin incision in the left flank was closed with metal clips. The 1 cm length of thread distal to the knot was trimmed so that no thread hung out of the vagina, but enough remained in the vagina to enable traction to be applied in those experiments in which it was desired to remove the thread.

In some experiments the carcinogen was maintained in position throughout the experiment, in other experiments the carcinogen was removed as soon as evidence of severe dysplasia or in-situ carcinoma was detected by exfoliative cytology. The removal was performed by cutting the thread at its subcutaneous anchorage and pulling it out via the vagina.

Anti-lymphocyte serum (A.L.S.)

The A.L.S. was supplied by Professor Van Bekkum of the Radiobiological Institute, T.N.O., Rijswijk, Holland, and had the following characteristics :

Cytotoxicity against lymph node cells 1 : 64

Cytotoxicity against thymus cells 1 : 1024

Mean survival time for allogeneic skin transplant with 1 ml A.L.S. : 14 days.

Total protein : 61.5 mg/ml

Albumen : 29.8 %

α Globulin : 25.1 %

β Globulin : 29.9 %

γ Globulin : 15.9 %

Details of the dose schedule are given under the experimental design heading.

Bacille Calmette-Guerin (B.C.G.)

Lyophilised B.C.G. was used. The B.C.G. was injected s.c. in a dose of 0.3 mg dry weight, suspended in 0.3 ml sterile pyrogen free water and contained $\pm 160,000,000$ living bacteria/ml. The time of injection varied according to the experimental groups and details are given under the experimental design.

Cytology

Cytological specimens were collected weekly starting 2 weeks before the implantation of the carcinogen and continued throughout the experiments. The specimen was collected using a Pasteur pipette shortened by breaking the narrow stem close to the body of the pipette. The sharp edges of the broken end were flame rounded. Normal saline, 0.5 ml, was installed into the vagina, withdrawn, and this was repeated three times. The fluid was transferred to a small centrifuge tube, lightly centrifuged and slides prepared from the deposit. The preparations were stained with Haematoxylin and Eosin, and Papanicolaou stains.

Histology

The uterus and/or tumour were removed en bloc. If no tumour was present the uterus was blocked intact and sectioned at 3 μ . Every tenth section was stained by Haematoxylin and Eosin. The lesions present in these cases were classified as Normal ; mild dysplasia ; severe dysplasia ; in-situ carcinoma and early invasive carcinoma. Early invasive carcinoma was defined as histological invasion with no demonstrable tumour formation. When tumours were present, blocks taken from the tumours were sectioned at 3 μ and stained with Haematoxylin and Eosin.

The Design and Aims of the Experiments.

There are two main groups of experiments :

- (a) Those in which the carcinogen remained in position throughout the experiment. These experiments were terminated six months after the implantation of the carcinogen (ST Experiments).
- (b) Those in which the carcinogen was removed at the first evidence of severe dysplasia or in-situ carcinoma as detected by exfoliative cytology, i.e. when the first cytologically malignant cells were detected. These experiments were terminated twelve months after the implantation of the carcinogen (LT Experiments).

ST Experiments

The experiments in which the carcinogen remained in position throughout were divided into the following groups :

- | | | |
|----------|------|---|
| ST Group | I) | A.L.S. or B.C.G. was started on Day - 14 relative to the day of carcinogen implantation, in randomly grouped mice. |
| ST Group | II) | In this group A.L.S. or B.C.G. was started on the day of carcinogen implantation, in randomly grouped mice. |
| ST Group | III) | This group consisted of mice with changes of in-situ malignancy or severe dysplasia. Individual mice were randomly grouped at the time the first cytologically malignant cells were detected, i.e. at the beginning of in-situ carcinoma or severe dysplasia in that mouse, and A.L.S. or B.C.G. given. |

ST Group IV) This group consisted of mice which showed cytological evidence of early invasion. Individual mice were randomly grouped at the time the earliest cytological signs of invasive carcinoma, without any palpable or visible tumour, were detected in that mouse, and A.L.S. or B.C.G. given.

The A.L.S. was given in an initial dose of 0.25 ml I.P. on days 0, 2, 5, and 7 and then 0.25 ml I.P. weekly for the subsequent three months.

B.C.G. was given in one dose of 0.3 mg dry weight, suspended in 0.3 ml sterile pyrogen free water.

N.H.S. was used as one control on a schedule and dosage identical to A.L.S. Saline injection 0.3 ml was given as a control for the B.C.G. experiments.

Cytology was performed weekly starting three weeks before carcinogen implantation.

The aims of these experiments were :-

- 1) To detect any variation in the time of first detection, by cytology, of malignant cells in groups ST I and ST II, i.e. to determine whether A.L.S. immunosuppression or B.C.G. immunostimulation accelerated or delayed the appearance of malignant cells, and thus the onset of in-situ carcinoma or severe dysplasia.
- 2) In groups ST I and ST II to determine if the administration of A.L.S. or B.C.G. affected the length of time from carcinogen implantation to the time of invasion as detected by cytology.

- 3) To detect any variation in the number of tumours produced by the administration of A.L.S. or B.C.G. when these agents are given at an identifiable stage of development, in the suggested progression from normal to carcinoma via either dysplasia or in-situ carcinoma, i.e. Group STI, at a time when the epithelium is normal, Group STII at implantation at the initial early inflammatory stage consistent with mild dysplasia, Group STIII on the detection of severe dysplasia or in-situ carcinoma, and in Group STIV on the detection of early invasive carcinoma.
- 4) If any variation occurs, to demonstrate histologically if the alteration in the number of tumours produced is, in the B.C.G. group, due to arrest at some stage in the progression from normal to carcinoma or is due to complete destruction of malignant cells and regression of the changes to normal, and in the case of A.L.S., if any enhancement of tumour growth occurs, is it due to A.L.S. acting preferentially at some stage in the early development of invasive carcinoma or is it a late acting phenomenon, affecting mainly the rate of growth of the tumour.

LT Experiments

The experiments, in which the carcinogen was removed on the first detection of malignant cells by cytological examination, were divided as follows :-

- | | |
|----------|---|
| LT Group | 1) In this group A.L.S. or B.C.G. was started in randomly grouped animals when the carcinogen was implanted. The carcinogen was removed when the first malignant cells were detected. |
|----------|---|

- LT Group 2) In this group, at the time the first malignant cells were detected by exfoliative cytology, the carcinogen was removed, the animals randomly grouped and A.L.S. or B.C.G. started.
- LT Group 3) This group consisted of animals that three months after the removal of the carcinogen showed cytological evidence of severe dysplasia or in-situ carcinoma only. The animals were randomly grouped and A.L.S. or B.C.G. started.
- LT Group 4) This group consisted of animals that three months after the removal of the carcinogen showed cytological evidence of early invasion only, without any palpable or visible tumour. The animals were randomly grouped and A.L.S. and B.C.G. started.

The controls in all the groups were animals receiving N.H.S. or saline injections in the same schedule and amounts as the A.L.S. or B.C.G. respectively. The A.L.S. and N.H.S. were given in an initial dose of 0.25 ml I.P. on days, 0, 2, 5 and 7 and then weekly for the next three months.

B.C.G. was given in one dose of 0.3 mg, dry weight suspended in 0.3 ml sterile pyrogen free water.

Cytology was performed weekly starting three weeks before carcinogen implantation.

The aims of these experiments were :-

- I In groups LT1 and LT2 to detect any variation in the time of first detection by cytology of malignant cells due to A.L.S. immunosuppression or B.C.G. immunostimulation, i.e. to the onset of severe dysplasia or in-situ carcinoma.

- II In groups LT1 and LT2 to determine if A.L.S. immunosuppression or B.C.G. immunostimulation affect the length of time from carcinogen implantation to the earliest evidence of invasion as detected by cytological examination.
- III To determine the effect of A.L.S. or B.C.G. on the progression or regression of pre-invasive lesions such as severe dysplasia or in-situ carcinoma, where these agents are given before removal of the carcinogen (Group LT1), at the time of removal of the carcinogen (Group LT2) or three months after removal of the carcinogen (Group LT3).
- IV To determine the effect of A.L.S. or B.C.G. on the subsequent course of early invasive carcinoma when these agents are given to animals showing cytological changes consistent with early invasive carcinoma only, three months after the removal of the carcinogen (Group LT4).
- V If A.L.S. or B.C.G. did have any effect to determine whether, in the B.C.G. groups, this is due to arrest of development at some particular stage or reversal to normal, and in the A.L.S. groups at what stage facilitation of tumour growth occurs.

Part II

ANTI-LYMPHOCYTE SERUM IMMUNOSUPPRESSION AND CHEMICAL CARCINOGENESIS.

INTRODUCTION : THE IMMUNOSUPPRESSIVE EFFECT OF A.L.S.

Although knowledge of the immunosuppressive action of anti-lymphocyte serum has been relatively recently acquired, similar sera have been used since the end of the nineteenth century. The first recorded applications by Metchnikoff ^(33, 34) were during the investigation of the cellular basis of inflammation. Since then sporadic reports have anticipated much of our current knowledge, such as the demonstration that A.L.S. agglutinated and killed lymphocytes in vitro, and that its cytotoxicity could be destroyed by heating the serum to 55°C. for thirty minutes ⁽³⁵⁾. Species specificity and the ability to cause a leukopenia in vivo were also demonstrated. Some of the early sera failed to discriminate among leukocytes ^(36, 37), but later lymphocyte specific anti-sera were demonstrated. Strong inhibition of the skin reactions of delayed hypersensitivity to tuberculin in guinea pigs by A.L.S. ⁽⁴⁰⁾ was shown, and these findings confirmed and extended to the prolongation of skin homograft survival and to the inhibition of auto-immune disease ⁽⁴¹⁾. With the combination of thoracic duct drainage and A.L.S., striking prolonged skin graft survival was obtained ⁽⁴²⁾. This demonstration was followed by work establishing A.L.S. as the most potent inhibitor of the experimental transplant rejection reaction ^(43, 44, 45, 46)

and as it is suggested that the mechanism of rejection of tumours is similar to the mechanism of transplant rejection ^(47, 48, 49), knowledge of the mode of action of A.L.S. and experience of its immunosuppressive activity under various experimental conditions are necessary for the critical understanding and interpretation of its effects on tumour growth.

A.L.S. injection in both animals and man is followed by a decrease in the number of circulating lymphocytes ⁽⁵⁰⁾, the lymphopenia being proportional to the injected dose ⁽⁵¹⁾ and appearing to be a specific effect of A.L.S. since it is only very slightly altered by adrenalectomy, and normal serum from the same donor species causes no lymphopenia, suggesting that stress from the serum injection plays no part. The lymphopenia appears to be due to a direct or indirect complement dependent cytotoxic activity ^(52, 53). The degree of lymphopenia appears to bear no direct relationship to the immunosuppressive activity ^(54, 55) although occasional dissenting observations have been made ⁽⁵⁶⁾.

The early descriptions of the histological modifications of the lymphoid organs by A.L.S. are somewhat confusing due to the differences in experimental conditions, animal species, quality and purity of the anti-serum and lack of distinction between specific and non-specific effects of A.L.S.

It is now generally agreed that the fraction of A.L.S. responsible for immunosuppression produces quite selective morphological alterations in the lymphoid organs ^(57, 58, 59, 60). The most prominent change is a marked

depletion of small lymphocytes from the para-cortical areas of the lymph node and the peri-arteriolar cuff of the spleen, i.e. the thymus dependent areas⁽⁶¹⁾. In contrast, germinal centres and medullary areas of the lymph nodes and follicular areas of the spleen are as a rule unaffected by A.L.S., and even after prolonged treatment the thymus is not significantly altered histologically^(61, 62).

After chronic A.L.S. administration however, lymphoid depletion can be obscured by hyperplasia and hypertrophy of germinal centres, medullary and follicular zones⁽⁵⁵⁾, and these changes were considered originally to be specific features induced by A.L.S.^(44, 45, 62, 63). Since, however, these changes are not observed in animals previously rendered tolerant to the foreign protein in the serum it is suggested that these features are non-specific and represent the morphological equivalent of the immune response of the recipient to the heterologous proteins constituting A.L.S.^(55, 64, 65).

That A.L.S. is a potent immunosuppressive has been affirmed by demonstrating its inhibitory effect on an extensive array of immune phenomena.

Although attempts to prolong skin allograft survival by A.L.S.⁽⁴⁰⁾ had previously been made, the first really significant results showing the immunosuppressive activity of A.L.S. in this situation were obtained by Woodruff and Anderson^(42, 66, 67) who were able to prolong the survival of first set skin allografts in rats across a strong histo-compatibility barrier. These findings have since been confirmed by many investigators and extended

to many species^(43, 68, 69, 70, 71) and skin allograft survival is the most common assay of immunosuppressive activity of anti-sera employed for experimental purposes^(72, 58).

The immunosuppressive effect is most evident when anti-serum is started prior to grafting, and can be further increased if the injections are continued after transplantation^(48,73,74), but the effect can also be apparent if the anti-serum is given after grafting^(75, 76).

The activity of A.L.S. as an immunosuppressant has also been confirmed in whole organ transplantation^(77,78,79) but the impression gained from the reports on organ transplants in larger mammals and humans is that the effects have not been as dramatic as those achieved with skin grafts in rodents⁽⁸⁰⁾. This may be due to genetic, technical or other complicating factors or to the proportionately smaller doses of A.L.S. which the larger animals tolerate and which are usually employed, although other factors such as humoral antibody damage⁽⁸¹⁾ may be more important in chronic allograft damage to whole organs. This may be of importance in the interpretation of results of A.L.S. administration on tumour growth since it is not known whether tumours behave as whole organs or as skin grafts.

In addition to the ability to prevent or delay the onset of cell mediated immunological reactions leading to the rejection of homografts and to arrest those already in progress, A.L.S. is also capable of inhibiting other cell mediated immune responses. An important example of this phenomenon

is the graft versus host (G.V.H.) reaction which results when immuno-competent cells, capable of reacting against the host, are injected into recipients incapable of rejecting them. In various experimental systems which leads to G.V.H. reaction, the immunosuppressive effect of A.L.S. has been demonstrated, e.g. the injection of parental strain cells into F1 hybrids (82, 83, 84), or the transfer of allogeneic lymphoid cells to neonatal or irradiated animals (85, 86).

Since the first report (39) there has been extensive confirmation of the ability of A.L.S. to suppress the dermal manifestations of delayed hypersensitivity (40, 85). Although inhibition of non-specific inflammation may play a role, the most likely explanation for the reported reduction of responsiveness and decreased mononuclear infiltrate at the site of challenge is the reduction of potentially reactive cells.

The induction and expression of delayed hypersensitivity to such antigens as tuberculin, ovalbumen, diphtheria toxoid, dinitro-chloro benzene and oxazolone (58, 87, 88, 89, 90) can also be prevented.

Whereas the immunosuppressive effect of A.L.S. on cell mediated responses is fairly general in most species, irrespective of the type of experimental condition, the same does not appear to be true for humoral antibody production where a number of factors, especially type and dosage of antigen, animal strain and species seem to play major roles in determining the final result.

An immunosuppressive effect of A.L.S. has been demonstrated on the antibody response to sheep erythrocytes in mice ^(91, 92), and rats ^(93, 94), to bacterial antigens in mice and dogs ^(43, 95), and to soluble proteins in mice and rats ^(87, 96). A.L.S. apparently interferes more readily with the humoral antibody response to thymus dependent antigens such as sheep erythrocytes and bovine albumen, although this is still disputed ⁽⁹⁷⁾.

The genetic constitution of the animals seems to have an important influence on these immunosuppressive effects; immune reactions in Sprague-Dawley rats are not inhibited by A.L.S. in contrast to Wistar rats ⁽⁹⁶⁾ and mice of the C.B.A. or A. strains are much more sensitive to A.L.S. than C₃H strain ⁽⁹⁷⁾. These findings are obviously of importance where Sprague-Dawley rats or C₃H mice are used in experiments designed to demonstrate the effect of A.L.S. in carcinogenesis, but they also suggest that other strains will have a greater or lesser response that must influence the interpretation of the results and warn against direct comparisons.

Doses of A.L.S. capable of suppressing the primary humoral response fail to have a pronounced effect on the secondary humoral response ^(98, 99) and other differences of effect on humoral and cellular responses were shown when a course of A.L.S. capable of eliminating the immune memory to a second set skin graft was unable to return a sensitised animal to virgin reactivity as regards B.S.A. ⁽¹⁰⁰⁾.

A number of hypotheses have been proposed to explain the immunosuppressive activity of A.L.S.

The first theory to be advanced was based on the in-vitro cytotoxic activity of A.L.S. and on the evidence of the observed lymphopenia and cellular depletion of lymphoid organs of A.L.S. treated animals ⁽⁶⁸⁾. Some observers also noted that the return to a normal immune reactivity, after discontinuance of injections, was coincident with the reappearance of normal levels of circulating lymphocytes and of normal histology on lymphoid organs ⁽¹⁰¹⁾. However, marked lymphopenia is not a constant feature, nor does its degree have any correlation with the level of immunosuppression induced, and it can be of much shorter duration than the period of immune suppression. Other procedures giving comparable or even greater lymphopenia, such as steroid injections or thoracic duct drainage ⁽¹⁰²⁾ do not induce equivalent immunodepression.

Another mechanism by which A.L.S. might act is that, by coating the lymphocyte membrane, A.L.S. might prevent any interaction between antigen and its specific receptor ⁽⁸³⁾ thus blocking an essential step of the immune response. However, this 'blindfolding' hypothesis could not be reconciled with the observations that immune incompetence after A.L.S. treatment persists in conditions where repeated lymphocyte divisions have occurred ⁽⁸³⁾, nor does it explain the non-suppressive activity of A.L.S. antibody fragments which also coat lymphocytes ⁽⁵²⁾.

It has also been suggested that A.L.S. might act by interfering with thymic hormone ^(86, 87), however, this theory appears untenable since the effects of A.L.S. and thymectomy are strongly synergistic ^(103, 104, 105).

'Sterile activation' was proposed on the basis of the finding of hypertrophic, hyperplastic lymphoid tissue, containing large numbers of blast cells, in A.L.S. treated animals ⁽⁷⁶⁾. This theory postulates that A.L.S. may induce a generalised, but non-immunologically productive, activation of lymphocytes, which would preclude any other immunological reactivity. Transforming ability is, however, shared by a wide variety of agents, none of which can compete with A.L.S. as an immunosuppressive ⁽¹⁰⁶⁾ and F(ab)₂ antibody fragments of A.L.S., which are immunologically inert, are able to produce blast transformation ⁽⁵²⁾. The increased number of blast cells after A.L.S. treatment are mostly the consequences of A.L.S. immunogenicity and this proliferative response is reduced in animals tolerant to A.L.S. ⁽⁵⁵⁾.

Evidence accumulating in the last few years leads to the conclusion that the mechanism of A.L.S. immunosuppressive activity is mediated through the inactivation of a selected subpopulation of cells, comprising the recirculating lymphocyte pool.

It is important to emphasise that the histological modifications induced by A.L.S. are the same as those resulting from thymectomy ^(107, 108), or thoracic duct drainage ⁽¹⁰⁹⁾, conditions that lead to depletion of the members of the long lived, recirculating thymus dependent lymphocyte pool, suggesting that A.L.S. has a similar effect.

There are also similarities in the characteristics of A.L.S. immunosuppression and that induced by thymectomy both of which impair the host's ability to mount a cell mediated response ⁽¹⁰⁸⁾, and thymectomy synergises

with A.L.S. therapy ⁽¹¹⁰⁾. The return to normal immune reactivity after A.L.S. treatment is slow and thymus dependant ⁽¹¹¹⁾, and lymphoid cells obtained from A.L.S. treated animals show a reduction in the elements bearing the θ antigen ⁽¹⁴⁷⁾ which is known to be a marker for thymus derived lymphocytes. They also lose their ability to respond to in-vitro P.H.A. ⁽¹¹³⁾ a property known to pertain to thymus derived cells ⁽¹¹⁴⁾.

Current knowledge therefore indicates that A.L.S. inactivates the members of the long lived thymus derived population with a slow turnover, continuously re-circulating via the lymph and blood through the lymphoid organs and periphery.

The action of A.L.S. in depleting the circulatory pool of long lived lymphocytes, containing the cells involved in the inductive, and effector, phases of graft rejection as well as the elements endowed with immunological memory for cellular reactivity, explains the major immunosuppressant effect on cell mediated responses, whereas only some humoral antibody responses are involved. The importance of this on the influence of A.L.S. on tumour formation, is that the most important inhibitory immune responses to tumours appear to be cell mediated ⁽¹¹⁵⁾ through thymus dependant lymphocytes, although this is not certain for the pre-malignant or pre-invasive stages. If immunological factors are of importance in the control of neoplasia, it follows that depression of this system, especially thymus-dependent (T) immune responsiveness, should facilitate tumour development, and it is well established in viral oncogenesis that thymectomy at or near birth leads

to an increased tumour incidence^(116, 117, 118). Similarly selective immunosuppression with antilymphocyte or antithymocyte serum markedly enhances the response to oncogenic viruses^(119, 120, 121).

Although the aetiological factors in the formation of the tumours are unknown, in patients 256 recipients of transplants have developed de novo malignancies⁽¹²²⁾, out of a suggested population at risk of 12,389 undergoing immunosuppression therapy after receiving a transplant⁽¹²³⁾. The most common epithelial lesions are the various carcinomas of skin and lips, 39%, and various stages of carcinoma cervix, 8%, of the total, respectively, with lymphomas forming 27%. These figures suggest that patients immunosuppressed for organ transplants have an increased risk of developing certain malignancies, and that host factors, possibly immunological, are involved.

The influence of immunosuppression on chemical carcinogenesis is less clear-cut than in viral oncogenesis. After the report that neonatal thymectomy significantly reduced host immunocompetence⁽¹²⁴⁾, it was claimed that thymectomized mice developed more skin tumours when treated with benzo (a) pyrene⁽¹²⁵⁾. However, others failed to observe any marked influence of thymectomy on the induction of skin papillomas or carcinomas following repeated skin painting with MC⁽¹²⁶⁾, and also failed to show any difference in skin tumorigenesis in intact or newborn thymectomized mice following initiation with an oral dose of urethan and promotion by skin-painting croton oil.

There are similar discrepancies in the reports on the influence of thymectomy on the carcinogenic response to subcutaneous injections of polycyclic hydrocarbons. It was suggested that neonatal thymectomy of C 57 BL mice rendered them more susceptible at the young adult stage to a subcutaneous dose (200 µg) of MC, as manifested by a reduction of latent induction period in thymectomized mice so that, at 14 weeks, 78% had developed sarcomas compared with 42% of sham-thymectomized control mice ⁽¹²⁷⁾. However, the final incidence of sarcomas in both groups was similar, approaching 100%, and there was no difference in the growth rates of the tumours evaluated from the time taken to develop from small (5 mm) nodules to a mean diameter of 15 mm.

An enhanced response to MC in inbred AKR and outbred CF 1 mice, but not inbred C₃H mice following neonatal thymectomy was also reported ⁽¹²⁸⁾. In the sensitive strains, tumours arose more rapidly whereas in AKR mice the final tumour incidence was increased from 70% to 100%

However, in other studies neonatal thymectomy of C 57 BL mice did not markedly enhance susceptibility to MC carcinogenesis, and the incidence and growth rates of tumours were not significantly different from those in controls ^(129, 130). Also, although thymectomy at 3 days of birth was reported to shorten latent induction periods ⁽¹³⁰⁾, this was not observed in the previous study ⁽¹²⁹⁾ where thymectomy was carried out at 24 hours and mice so treated had proven immunoincompetence as evidenced by their failure to reject skin allografts. The most consistent feature arising from these studies was the increased immunogenicities of the sarcomas arising in thymectomized mice as

qualitatively revealed by showing that resistance could be induced in syngeneic mice against transplants of 9/10 tumours arising in thymectomized mice, whereas only 5/10 tumours from control, sham-thymectomized mice had comparable immunogenicities (129).

In the second of these studies comparison of the immune response elicited against tumours arising in thymectomized and non-thymectomized mice, by the rejection of a standard challenge dose of tumour cells, (10^3), in normal syngeneic mice immunized by tumour excision (130), indicated significantly increased immunogenicities in tumours arising from a dose of 250 μ g of MC in thymectomized mice compared with sham-operated controls. A 4 - fold larger dose of carcinogen, however, failed to enhance tumour immunogenicity in thymectomized mice, suggesting that the variable influence of thymectomy on MC - carcinogenesis may be due to the immunosuppression induced by the carcinogen which is known to be dose dependent (131).

Neonatally thymectomized mice showed increased sensitivity to chemical carcinogenesis in studies on lung adenoma formation after treatment with 7, 12 - dimethylbenz (a) anthracene or urethan (132, 133), and restoration of the immunological competence of the thymectomized mice, by repeated thymus implantation, reduced the sensitivity to urethan, the number of lung adenomas per mouse being similar to that of intact controls (134).

Reports of the effects of anti-lymphocyte serum (A.L.S.) immunosuppression on chemical and physical carcinogenesis show similar variation in the results. Since these reports are of most pertinence to the experiments reported in this thesis they are given here in some detail.

In mice, C.B.A. strain , in which sarcomas were induced by 1.0 mg M.C.A. injected subcutaneously, Rabbit A.L.S. given in a dosage of 0.25 ml twice per week, starting 6 days before administration of the carcinogen, increased the incidence of tumours and decreased the latent period ⁽¹³⁵⁾.

In C57 BL/6 J mice in which tumour induction was by 2.0 mg M.C.A. subcutaneously, 0.25 ml. Horse A.L.S. given twice per week for twelve weeks, starting 14 days after administration of the carcinogen, increased the incidence of tumours and decreased the latent period ⁽¹³⁶⁾.

Tumours induced by 0.05 mg M.C.A. I.C. in C.B.A. x C57 BL/F, Hybrids had a decreased latent period and an increased growth rate when the mice were treated by either Horse or Rabbit A.L.S. given I.P. in doses of 0.5 ml once per week for five weeks or 1 ml twice per week for one week followed by 1 ml once per week for eight weeks, starting on the day of induction of carcinogenesis ⁽¹³⁷⁾.

In these three experiments the dosage of A.L.S. used caused sufficient systemic immunosuppression to delay allograft rejection.

In C₃Hf and I strain mice, a strain effect of anti-thymocyte globulin (A.T.G.) on tumour formation was demonstrated. Tumours were induced by the administration of 0.2 to 2 mg M.C.A. subcutaneously, and 0.25 ml of a 1:4 dilution of Rabbit A.T.G. given I.P. on the day of induction and thereafter 3 times in the first week, followed by 0.25 ml once per week to the end of the experiment. The incidence of tumours in strain I was increased, but there was no effect on the C₃Hf strain mice ⁽¹³⁸⁾. The immunosuppressive

effect of the A.T.G. regimen was demonstrated in this experiment by decreased graft v host reaction, decreased mixed lymphocyte culture response and decreased anti-SRBC haemagglutinin titres. This finding is in keeping with the low sensitivity of C₃H mice to A.L.S.⁽⁹⁷⁾, but may also merely reflect the very high sensitivity of C₃H strain mice to the carcinogenic effect of M.C.A. or the marked sensitivity of C₃H mice to the immunosuppressive effect of M.C.A. over which any effect of A.L.S. might be minimal⁽¹³⁹⁾.

An effect of the amount of carcinogen applied was demonstrated by implanting subcutaneously into (C 57 BL/6 x BALB/C) hybrids M.C.A. impregnated discs containing 0.03 or 0.15 mg M.C.A. Combined adult thymectomy and A.L.S. (0.25 ml on days - 1, + 1,3 and 5 in relation to the carcinogen implantation, day 0) resulted in increased tumour incidence with low dose M.C.A., but no effect with the higher dose M.C.A.⁽¹⁴⁰⁾. In these experiments the immunosuppressive effect of the A.L.S./thymectomy regime was demonstrated by delayed allograft rejection and increased growth of M.C.A. tumour transplants. These results might reflect persistence of the effect of the high dose of M.C.A. at the time the immunosuppressive effect of A.L.S. was diminishing.

Syrian hamsters, treated with Rabbit A.L.S. 1 ml I.P. initially, followed by 0.45 ml three times per week for nine weeks, starting 6 weeks after the carcinogen application, had a larger number of tumours induced by the application of 0.5% D.M.B.A. on the cheek pouch than control animals. The application was three times weekly. Histologically the tumours induced

in the A.L.S. group were more undifferentiated and invasive than those of the control group. Unfortunately in this experiment the immunosuppressive effect of the A.L.S. used was only tested in vitro, and the in vivo effect of the regimen was not tested. Nor was the effect of N.R.S. demonstrated ⁽¹⁴¹⁾.

In a similar experiment Rabbit A.T.S., 0.25 ml S.C. three times per week in one group, and 0.1 ml S.C. three times per week in a second group, beginning 1 or 8 days before the application of 0.5% D.M.B.A. to the hamster's cheek pouch, resulted in a shortened latent period and an increased number of tumours compared to a control group. The carcinogen was applied three times per week ⁽¹⁴²⁾. In this experiment, as in the preceding one, the in vivo immunosuppressive effects of the A.T.S. regime was not demonstrated nor was N.R.S. used in one of the control groups.

Rabbit A.L.S., 0.5 ml I.P., three times per week, in an experiment using the carcinogenic effect of painting 0.5% D.M.B.A. in heavy mineral oil onto the buccal pouch of outbred Golden Syrian hamsters, resulted in a significant increase in the number of carcinomas compared to control groups receiving either N.R.S. or no treatment. The carcinogen was painted onto the buccal pouch three times per week for 10 weeks. The efficacy of the A.L.S. therapy was determined by its ability to delay rejection of skin allografts and by its cytolytic effect on hamster thymocytes ⁽¹⁴³⁾.

Objections to these three experiments can be raised since the hamster buccal pouch is an immunologically privileged site (ref.) and results obtained using the immune manipulation of tumours induced in this site may not be generally applicable.

Studies investigating the influence of A.L.S. - induced immuno-suppression on different stages of skin carcinogenesis by various carcinogens in mice have been reported. In a group of S.W.R. mice using topical application of 1.5% D.M.B.A. to the skin followed by promotion with croton oil twice per week to the end of the experiment, as the means of tumour induction, treatment with 0.5 ml Rabbit A.T.S. once per week had no effect on papilloma formation, but resulted in decreased papilloma regression and an increased progression from benign to malignant tumours⁽¹¹⁾. The immunosuppressive effects of this dosage of A.T.S. was demonstrated by delayed tumour allograft rejection.

Using ultra violet (UV) light, 2.82 mJ/mm^2 five times per week for 30 weeks, as a source of physical carcinogenesis on the skin of H-1 mice, it could be demonstrated that A.L.S. given S.C. at a dosage of 0.1 ml twice per week for 20 weeks starting 14 days before exposure to the UV light, resulted in greatly increased tumour incidence, increased numbers of tumours per mouse and a shortened latent period⁽¹⁴⁴⁾. Unfortunately in this experiment the immunosuppressive effect of the A.L.S. was not demonstrated nor were N.R.S. controls used so that although the magnitude of the effect of A.L.S. on UV light carcinogenesis was considerable, the conclusion that this was due to the immunosuppressive effect of A.L.S. alone cannot be sustained in this experiment.

In DBA/2 mice, receiving $\pm 0.3 \text{ mg}$ M.C.A. applied to the skin three times per week for four weeks, Rabbit A.T.S., 0.5 ml I.P. twice per week in the 1st and 3rd weeks after the start of carcinogen application, caused a slight decrease in the latent period of skin papillomas, but had no effect

on the leukaemia incidence ⁽¹⁴⁵⁾. The immunosuppressive effect of the A.T.S. was demonstrated by delayed allograft rejection.

In an attempt to bypass the systemic immunosuppressive effect of the M.C.A. carcinogen, after the application of the M.C.A. to the skin of BALB/C mice the skin was then transplanted to other BALB/C mice. The mice with the skin graft were then treated with Rabbit A.L.S. 0.25 ml I.P. daily for 7 days starting on the day of grafting. This resulted in a marked decrease in the regression of the papillomata and in this experiment histological evidence was also provided that the regression of skin papillomata can be immunologically mediated ⁽¹⁴⁶⁾.

Incipient tumours were produced by the administration of 5% 3 - methyl-cholanthrene pellets each pellet containing 0.15 mg, in normal and immunodepressed BALB/C mice. Immunodepression was achieved by thymectomy plus a regimen of rabbit anti-mouse lymphocyte serum. The A.L.S. was given 0.25 ml on days -1, +1 and +3 relative to the implantation of the M.C.A. and once weekly thereafter. After two months, half the animals in each group (normal and immunodepressed) were subjected to full thickness autografting of the area containing the M.C.A. pellet. The grafts were lifted from their graft bed and then replaced in their original position and secured by metal clips. No tumours developed in the autografted sites of normal mice. Normal mice with non-grafted sites and both groups of immunodepressed mice had significant tumour development ⁽¹⁴⁷⁾. The A.L.S. immunosuppressive potency was demonstrated against minor and major H-2 incompatible graft combinations. Unfortunately, N.R.S. was not used in a control group. This suggests that while they remain undisturbed, the developing lesions do not

stimulate an immune response which will eliminate the developing malignant cells, but that the inflammatory changes which are caused by grafting are sufficient to allow access of immune competent cells and that the resulting response will eliminate the developing malignant cells.

Repeated administration of rabbit anti-mouse lymphocyte serum (A.L.S.) 0.1 ml I.P. weekly for first 10 weeks of life to Charles-River mice given 100 µg 7, 12 dimethylbenz (a) anthracene at birth, greatly reduced the mean survival time of mice, markedly increased the number of tumours, and shortened the latency period, compared to control groups receiving N.R.S. in similar amounts, or control groups receiving only D.M.B.A. (148).

Rabbit A.L.S., 0.05 to 0.25 ml given once I.P., 0 to 50 days after birth, increased the incidence of malignant lymphomas, induced by 100 µg D.M.B.A. given S.C. in Charles River strain mice. In this experiment the immunosuppressive effect of the A.L.S. regimen was demonstrated by decreased plaque forming cell response to S.R.B.C. (149). However, the mice controlled by N.R.S. in this experiment had an equally increased incidence of malignant lymphomas suggesting that the effect of A.L.S. was not related to its immunosuppressive activity but was a non-specific effect due to the antigenic stimulus of the rabbit serum.

In a similar experiment, Rabbit A.L.S. given I.P. in a varying regimen, either 0.1 ml once per week for 10 weeks after birth, or 0.05 ml or 0.1 ml daily for seven days or the first few weeks after birth, increased the incidence of lymphomata or lung tumours in Charles River strain mice given 100 µg D.M.B.A. subcutaneously. The immunosuppressive effect of the A.L.S. was

again demonstrated by a decreased plaque forming cell response to S.R.B.C.⁽¹⁵⁰⁾. Unfortunately, the effect of a control group receiving N.R.S. was not investigated in the experiment. It is of interest, however, that the different schedules of A.L.S. administration selectively affected the production of either lymphomas or lung tumours.

Rabbit A.T.S., 0.5 ml I.P. on days -6, -4, -2, 0, or 0.5 ml given once per week for four weeks then six bi-monthly injections beginning day -1, relative to day of starting the carcinogen, increased the incidence and reduced the latent period of plasmacytomas induced in BALB/c mice by 0.5 ml mineral oil given I.P. once per month for 3 months⁽¹⁵¹⁾. The immunosuppressive effect of the A.T.S. was demonstrated by decreased graft versus host reaction and delayed allograft rejection.

Rabbit A.T.S., 0.2 ml twice per week s.c. from day -20 to day +10, relative to day of giving carcinogen, decreased the latent period and increased the incidence and number of lung adenomas per mouse induced by 0.5 mg/gram Urethan I.P. in Swiss mice. Similar results were obtained in SWR/J mice with identical carcinogen, dose, and rate of administration, but in which the Rabbit A.T.S. was given as 0.1 ml s.c. three times per week from week -4 to week +4⁽¹³⁴⁾. The immunosuppressive effect was demonstrated by delayed tumour allograft rejection, decrease in haemagglutinin and haemolysin titres and decreased peripheral blood leucocyte count during the experiment.

Horse A.T.S., 0.5 ml I.P. on day -3 and 0.25 ml twice per week for 30 weeks increased the incidence of bladder tumours, but had no effect on neural tumours in CDF rats given M.N.U. 10 mg/Kg twice per week for

nine weeks I.V. ⁽¹⁵²⁾. The immunosuppressive effect of the A.T.S. regimen was demonstrated by delayed allograft rejection during the experiment.

The incidence of pre-neoplastic liver lesions was increased, in rats receiving D.E.N.A. as carcinogen at a dose of 8 mg/Kg per day for six weeks, by the injection of Rabbit A.L.S. 1 ml on day 0 followed by 0.5 ml daily for six weeks I.P. ⁽¹³⁾. The immunosuppressive effect of this regime was not tested and neither was the strain of rats used or the route of administration of the carcinogen given.

In a similar experiment rats received either a single dose of 10 mg of diethylnitrosamine (D.E.N.A.) /Kg 24 hours after partial hepatectomy, followed by 2 ml of horse anti-rat A.L.S. once weekly for six weeks or a carcinogenic regimen of 8 mg D.E.N.A./Kg/day together with 2 ml A.L.S. once weekly for six weeks. No difference in the number and size of the pre-cancerous lesions could be detected from those occurring in the respective controls receiving N.H.S. / or no serum. However, when rats subjected through to the second procedure were further left untreated the rate of death from liver tumours in the A.L.S. group was significantly enhanced as compared with the control groups ⁽¹⁵³⁾. If the effect of the A.L.S. on the neoplastic process is mediated by immune suppression then the results of this experiment indicate that the cells participating in liver carcinogenesis become immunogenic to the cellular immune system beyond the pre-cancerous stage of development.

In contrast to these experiments which demonstrated some effect of A.L.S. on chemical and physical carcinogenesis, an extensive analysis of A.L.S. on MC carcinogenesis in several strains of mice, reported that no

effect on the incidence or latent period in most strains of mice could be demonstrated by the administration of 0.25 ml Horse or Rabbit A.T.S. four times per week, for one week, followed by 0.25 ml once per week thereafter, the administration beginning on day 0 or day +50, relative to the injection of the carcinogen. The carcinogen was M.C.A. given intramuscularly. This experiment used a large number of different strains of mice (B10.A., B10.D2., B10.129., B10.C₃H., B10.c., C57Bl/10., A.By., A.SU., HTH/AO., A/Wy Sn.) and in most strains there was no effect on the latent period or tumour incidence ⁽¹⁴⁾. The immunosuppressive effect of the A.L.S. was demonstrated by concurrent tumour allograft rejection.

Nor could any effect be demonstrated by the injection of 2 ml A.L.S., I.P. on the 100th day after the carcinogen on latency or incidence of tumours induced in Lewis rats after the injection of 10 mg M.C.A. s.c. ⁽¹⁵⁴⁾. In this experiment an immunosuppressive effect was only checked by a decrease in the lymphocyte count.

No effect on the incidence of tumours was shown in C57BL/6J mice given 2.0 mg M.C.A. subcutaneously following the administration of Rabbit A.L.S. s.c. in a regimen of 0.1 ml three times per week for two or six weeks or continuously to the end of the experiment ⁽¹⁵⁵⁾. The immunosuppressive effect of the A.L.S. could be demonstrated by delayed allograft rejection. It should be noted, however, that the amount of carcinogen used was very high and the incidence of tumours approached 100% suggesting the carcinogenic effect would overwhelm and obscure any effect of A.L.S.

In Buffalo strain rats, Goat A.T.S. 1.5 ml daily for thirty days starting day -2 did not affect the incidence of mammary tumour development after they had received D.M.B.A. 10 mg per week for four weeks ⁽¹⁵⁶⁾. The immunosuppressive effect of the Goat A.T.S. was shown by delayed allograft rejection.

PART III

B.C.G. IMMUNE STIMULATION AND CHEMICAL CARCINOGENESIS.

Introduction

B.C.G. was originally isolated by Calmette and Guérin at the Pasteur Institute of Lille through the progressive attenuation of a virulent strain of *Mycobacterium Bovis*.

Later the stimulating effect of B.C.G. on the immune response to antigens^(157,158,159), and its remarkable stimulating effect on the reticulo endothelial system⁽¹⁶⁰⁾ were discovered. When stimulation of the reticulo endothelial system with Zymosan was demonstrated to be associated with prevention of tumour growth⁽¹⁶¹⁾ there rapidly followed the investigation of, and demonstration of, the inhibitory effect of B.C.G. on tumour growth^(7, 162, 163, 164).

Experience gained from the use of B.C.G. in protection against tuberculosis.

Before assessing the reported effects of B.C.G. on carcinogenesis, it is of interest, and educative, to consider what has been learned from the extensive studies of its use in protection against tuberculosis, since even here the results are by no means conclusive, and the variations and causes of the variations, may be relevant to the interpretation of the results of investigations of the effect of B.C.G. on tumour formation.

All cultures of B.C.G. originated from the original strain derived by Calmette and Guérin, but due to the differences in preparation, culture methods and genetic drift the strains are not now identical and B.C.G. as

currently employed has the disadvantage of variability in strain characteristics leading to substantial differences in biological behaviour⁽¹⁶⁵⁾. The results of B.C.G. immunisation programmes against tuberculosis have been variable, and the variability appears to be explicable mainly by differences in the potency of the B.C.G. vaccines used⁽¹⁶⁶⁾. Another suggested explanation has been that the degree of protection provided by B.C.G. is greatest in areas where the incidence of tuberculosis is high presumably due to a booster effect of frequent contact with stimulating mycobacterial antigens⁽¹⁶⁷⁾. This corresponds with studies from a community free from tuberculosis and other mycobacterial infections where a steady and substantial decrease in the incidence of skin sensitivity could be demonstrated after B.C.G. vaccination⁽¹⁶⁸⁾. In contrast to this, in another study, tuberculin sensitivity was unchanged two months to five years after B.C.G. immunisation and there was little difference in skin sensitivity resulting from the use of eleven different B.C.G. vaccines⁽¹⁶⁹⁾. Variations in potency of vaccines derived from different strains have, however, been demonstrated in laboratory animals⁽¹⁷⁰⁾, and also in studies in different human populations^(171,172), the most immunogenic strain being the Pasteur, and the least immunogenic the Glaxo strain.

Further variable characteristics of different strains include : viability in rabbit, mouse and guinea pig spleens : Jensen intradermal test in guinea pigs ; allergenicity in animals ; protection against tuberculosis in mice and guinea pigs ; size of bacilli ; colony morphology and enzyme characteristics and phenotype variations⁽¹⁶⁵⁾.

Different types of preparation of B.C.G. have markedly different effects on the type of immune response of the recipient. Fully viable B.C.G. preparations (log phase cultures) caused blast transformation of lymphocytes, and cell proliferation with little histological evidence of antibody response, whereas lyophilised commercial preparations, which had low viability and a high concentration of soluble antigenic material, induced a strong antibody response⁽¹⁷³⁾.

In view of the variable characteristics exhibited by living strains of B.C.G. the use of fractions of cell components has been advocated in an attempt to obtain standardised preparations. Extensive reviews of the properties of non-living B.C.G. vaccines suggested that the bias in favour of living vaccines was unjustified since many experiments contained substantial methodological errors, the most common being the low dosage of non-living B.C.G. not allowing for the effect of multiplication of the living organisms^(174, 175). Vaccines of non-living B.C.G. components have had substantial activity in increasing resistance against isologous tumours, and micro-organisms, and have the advantage that at least some of the hazards of living vaccines are avoided⁽¹⁷⁶⁾.

These variations in biological activity of B.C.G. make comparisons of the effect of different B.C.G. programmes on the protection against tuberculosis difficult to evaluate and similar problems with strain and test variations are likely to make equally difficult the interpretation and comparison of results of B.C.G. immunotherapy or immunoprophylaxis from different centres using different B.C.G. vaccines and different schedules of vaccination, especially in these experiments where insufficient experimental detail is given.

The mechanism of immunity to tuberculosis is not known precisely and a variety of immune responses occur after B.C.G. vaccination, but the two predominant cell types involved in B.C.G. induced immunity to tuberculosis, the lymphocyte and the macrophage, appear also to be those involved in B.C.G. augmented tumour immunity. Both B.C.G. and the presence of a growing tumour will transiently increase the number of bone marrow monocytes, and, at a time when the effect of either has disappeared, the combination of the two will continue to produce significantly greater numbers of monocytes ⁽¹⁷⁷⁾. This suggests that analysis of changes in these cells, quantitative or qualitative, produced by B.C.G. vaccination against tuberculosis, or B.C.G. macrophage stimulation against other organisms, may be of importance in defining more precisely the changes of importance in the B.C.G. induced response to neoplasia.

The demonstration of delayed hypersensitivity (D.H.) by a positive skin test to purified protein derivative (P.P.D.) is recognised as signifying resistance to tuberculosis ⁽¹⁷⁸⁾ although subject to considerable objective variation.

In vitro confirmation of the role of delayed hypersensitivity in immunity to tuberculosis ⁽¹⁷⁹⁾, has been provided by the demonstration that the degree of D.H. of skin to purified protein derivative (P.P.D.) of tuberculin, correlates with the logarithm of the lymphocyte transformation index. Further, it has been shown that skin test negative individuals have lymphocytes with low affinity receptors for tuberculin, while skin test positive individuals have lymphocytes with high affinity receptors ⁽¹⁸⁰⁾. Lymphocyte transformation with high doses of antigen is therefore possible, even though the skin tests are negative.

It was formerly believed that cellular immunity in tuberculosis was due to long-lived macrophages gradually being activated and destroying the bacilli⁽¹⁸¹⁾. Using the presence of B - galactosidase as a macrophage marker, and tritiated thymidine (H^3 -T) to assess D.N.A. synthesis, most of the mononuclear cells in tuberculous lesions in rabbits were shown to be recent immigrants and local division did not contribute substantially to their number⁽¹⁸²⁾. The presence of D.H. correlated with monocyte entry into the tuberculous lesions. Both the tissue monocyte (macrophage) and the circulating monocyte have been shown to produce a substance highly active in stimulating human bone marrow granulocytic and mononuclear cell production in vitro⁽¹⁸³⁾. These properties are particularly important, as local cellular division of macrophages in B.C.G. lesions cannot explain this high local concentration.

Macrophages stimulated by B.C.G. have increased ability to inhibit the growth of certain intracellular parasites^(184, 185). A quantitative effect has been shown in that the clearance of *Listeria monocytogenes* from the blood was increased substantially in mice receiving 4×10^6 viable B.C.G.; 4×10^3 B.C.G. resulted in only a slight clearance effect⁽¹⁸⁶⁾. The resistance of liver and spleen to *Listeria monocytogenes* infection correlated with the maximal number of living B.C.G., and rose in parallel with the development of cutaneous D.H. With re-infection by B.C.G. there was an increase in resistance, i.e. increased clearance of organisms from blood, and killing in liver and spleen. Local multiplication was important in producing this effect, since heat-killed B.C.G. was the least effective and

only slightly better effect was noted in animals receiving isoniazid with B.C.G. These results correlated with the development of cutaneous D.H. and with cytological changes in the peritoneal macrophages suggesting increased metabolic and enzymatic activity.

Immune aspects of B.C.G. and tumour growth.

In carefully performed studies a weakly antigenic, immunogenic, hepatocarcinoma was cutaneously transplanted to guinea pigs. After metastases to regional lymph nodes had occurred the guinea pigs were treated by intra-lesional injection of B.C.G. Tumour rejection was associated with B.C.G. mediated granulomatous reaction at both the tumour site and in the regional lymph nodes in which the predominant cell involved appeared to be the macrophage ⁽¹⁸⁷⁾. This is in agreement with previous *in vitro* studies showing that ascites tumours ⁽¹⁸⁸⁾ and transplantable lymphomas may be destroyed by macrophages. In these experiments the B.C.G. organisms were found to be associated with macrophages and not in direct contact with tumour cells, suggesting that it is stimulation of macrophage activity, rather than any effect of the B.C.G. on the tumour cells, that is of importance.

Support for the importance of macrophage-mediated tumour is provided by recent experimental evidence on activated macrophages ^(189, 190, 191, 192, 193). Macrophages activated by peptone injections or by nematode infection inhibit the growth of Walker carcinosarcoma cells in rats, if the macrophages are activated 5 days before tumour cell injection ⁽¹⁹⁴⁾. Since the effect was not decreased by irradiation, it implies that functionally intact lymphocytes are not required. The effect is lost ⁽¹⁹⁵⁾ when macrophages are incubated with a serum 'blocking' factor.

In a study using several rat tumours of varying immunogenicity, nude athymic mice (nu/nu) were injected subcutaneously with a suspension of tumour cells or tumour cells plus B.C.G.⁽¹⁹⁶⁾. Progressive growth was observed in all control animals, but in the animals receiving B.C.G., complete inhibition of tumour growth occurred. These studies suggest that the B.C.G. suppression of tumour growth is not effected by a lymphocyte mediated response, but that it is mediated by macrophages.

Mouse macrophages activated by toxoplasma, were able to kill cells from the syngeneic sarcoma K.H.T., and the B.A.L.B./c mammary adenocarcinoma E.M.T.-6.⁽¹⁹³⁾

Other intracellular micro-organisms for example, *Besnoitia* Jellisoni and *L. monocytogenes* also activate macrophages. In addition both endotoxin and double stranded R.N.A. cause murine peritoneal macrophages to become cytotoxic to certain neoplastic cell lines⁽¹⁹⁷⁾, but materials such as starch and thioglycollate media produce inflammation, but no macrophage activation.

Activated macrophages have little or no cytopathic effect against non-transformed cells, which do not cause malignant growth when inoculated with syngeneic hosts and have a limited life span in vitro⁽¹⁹²⁾.

The presumed active site of the activated macrophage is the cell membrane since the macrophages adhere to the tumour cells, and after 24 hours of contact between, for example, macrophages and lymphoma cells, irreversible damage to the tumour cells occurs and if the lymphoma cells are removed after contact, the macrophages are unable to damage subsequently added lymphoma cells. Phagocytosis was not involved in growth inhibition and occurred only after tumour cells were disintegrating.

Using differential centrifugation, a sedimentable sub-cellular fraction was obtained from B.C.G. - infected mouse livers which lysed mastocytoma cells in vitro more rapidly than the corresponding fraction from the livers of normal mice. This observation suggests that B.C.G. was able to augment the enzymatic activity of mononuclear cells in the liver⁽¹⁹⁸⁾.

While activated macrophages may non-specifically kill tumour cells by cell surface activity, immunity to subsequent challenge is specific⁽¹⁹⁹⁾, and it has been shown that immunisation of guinea pigs with B.C.G. plus one of 2 antigenically distinct hepatocellular carcinomas did not protect against the other hepatocellular carcinoma⁽²⁰⁰⁾.

Specificity has also been shown for B.C.G. induced granulomatous responses⁽²⁰¹⁾, the accelerated granulomatous response occurring only when B.C.G. sensitised rabbits are challenged with B.C.G., but not when other granuloma inducing organisms are used.

Current evidence suggests that the mechanism of B.C.G. anti-tumour activity is a 2 step phenomenon. The first step involves recognition of B.C.G. by the host defence system and is immunologically specific ; the second step is a granulomatous reaction in which macrophages are mobilised and activated by lymphocyte activation products⁽²⁰²⁾, produced by specifically immune T cells reacting with antigens of B.C.G., and the activated macrophages subsequently effect the non-specific destruction of the neoplastic cells. Subsequent specific immunity may be due to increased numbers of immunologically competent lymphocytes being available for activation by tumour specific antigens.

However, lymphocytes and serum ⁽²⁰³⁾ from B.C.G. treated B.A.L.B./c mice were cytotoxic to S91 melanoma cells, but not to mammary carcinoma, 3M.C. induced sarcoma or fibroblast target cells from B.A.L.B./c mice. These results suggested a specific B.C.G. induced protection against S91 melanoma cells, possibly due to sharing cross reacting antigens. Similar findings were also reported of an antigenic relationship between B.C.G. organisms and line 10 cells ⁽²⁰⁴⁾. These findings were extended by the demonstration of cell surface antigens on line 10 hepatocarcinoma by immune electron microscopy using xenogeneic and syngeneic tumour specific antibodies and syngeneic B.C.G. induced antibody, showing that there are common antigens between line 10 hepatocarcinoma cells and B.C.G. organisms. This specificity was further confirmed ⁽²⁰⁵⁾ by blocking experiments and absorption of specific antibody to B.C.G. by the tumour cells.

Antibodies in sera from rabbits immunised with line 10 cells and from rabbits immunised with B.C.G. both bound B.C.G. and line 10 antigens. Inhibition experiments confirmed the specificity of the reactions and also showed that antigenic components are shared by B.C.G. and extracts of Rous sarcoma cells ⁽²⁰⁶⁾. Whether the shared antigens are identical with tumour specific antigens and whether they have a function in the outcome of immunotherapy remain open questions.

Inbred strain -2 Sewall Wright guinea pigs were treated and cured of line 10 hepatocarcinoma by the administration of sub cellular components of *Mycobacterium bovis* strain B.C.G.; 53% of the newly born offspring

resisted repeated challenges with the tumour. 23.5% of offspring of mothers given B.C.G. only, resisted tumour challenge and the disease was attenuated in many others ⁽²⁰⁷⁾. All control offspring from normal untreated mothers succumbed to the tumour. Resistance was associated with delayed cutaneous hypersensitivity reactions to tumour and mycobacterial antigens. In vitro, it has been shown that lymphocytes from both B.C.G. immunised guinea pigs and tuberculin - sensitive humans are more efficient in lysing mastocytoma cells coupled with P.P.D. than non-immune lymphocytes ⁽²⁰⁸⁾. However, this does not appear to be the situation in vivo since the B.C.G. organisms and tumour cells are not in contact.

Complement dependent cytotoxic antibody to tumour cells has been demonstrated in the sera of strain - 2 guinea pigs whose tumours had been cured by B.C.G. treatment ⁽²⁰⁹⁾.

This cross reactivity of B.C.G. and antigens on tumour cells may also be related to the ability of B.C.G. under certain circumstances to enhance tumour growth since it is known that B.C.G. increases antibody production and enhances cell mediated immunity. The enhancement of tumour growth has been attributed to the formation of blocking antibody ^(210, 211, 212, 213), or antigen-antibody complexes ^(214, 215), but lymphocytes have also been shown to have stimulatory activity ^(216, 217). If the antibodies produced against B.C.G. react with tumour antigens to produce antigen-antibody complexes these may have a blocking effect on cell mediated cytotoxicity, or lymphocytes sensitised to B.C.G. might respond to tumour

antigens by a stimulatory effect on the tumour cells. The first effect could be of special importance in those experiments using commercial lyophilised B.C.G., with its high soluble antigen component, which tends to produce antibody rather than a cellular response ⁽¹⁷³⁾.

Immunological stimulation of tumour growth has been observed following the inoculation of B.C.G. into man and laboratory animals. B.C.G. given I.V. to rats enhanced rather than suppressed, pulmonary growth of transferred cells from several weakly immunogenic tumours ⁽²¹⁸⁾. Facilitation of tumour growth by B.C.G. was also demonstrated using a carcinogen-induced mammary tumour in rats ⁽²¹⁹⁾.

In guinea pigs, the growth of intra-muscularly transplanted hepatocellular carcinoma could not be suppressed by intra-lesional injection of B.C.G. and in animals previously vaccinated with B.C.G. tumour growth was accelerated ⁽²²⁰⁾.

Acceleration of malignant melanoma in humans has been reported following the intra-lesional administration of B.C.G. ^(221, 222). In one case serum blocking factors developed ⁽²²¹⁾ and in another case the development of stimulatory lymphocytes was correlated with the appearance of new lesions ⁽²²²⁾.

In some circumstances the enhancing effect of B.C.G. appears to be dose dependant. Groups of C57Bl/6J mice pretreated with either high dose (0.5 mg) B.C.G. or low dose (0.005 mg) B.C.G. were then injected with B16 melanoma cells. In mice pretreated with the high dose the tumour grew at an accelerated rate, whereas with the lower dose the tumour grew at a

reduced rate compared with control mice ⁽²²³⁾. This enhancement may be due to the more rapid induction and a greater intensity of the immune response following injection of increased doses of B.C.G.

It should be noted however, that two phenotypes of the Montreal strain of B.C.G. derived by culture of one strain of B.C.G., had opposite effects on the early development of subcutaneous transplants of epithelial carcinoma ⁽²²⁴⁾ one accelerating, the other inhibiting, tumour growth.

Another aspect of B.C.G.'s action is the ability of B.C.G. to induce the production of "unblocking sera". Blocking sera contain substances, Ag-Ab complexes, abrogating in vitro observed cell mediated lymphocyte cytotoxicity; unblocking sera, when added to blocking sera, will reverse this effect. B.C.G. primed rats and rabbits immunised with tumour cells under certain circumstances ⁽²²⁵⁾, were able to produce "unblocking" sera, probably due to alteration in the ratio of Ag : Ab produced when "unblocking serum" was added to the "blocking serum".

B.C.G. and the treatment of tumours.

Attempts have been made experimentally to treat established tumours either with B.C.G. alone or with B.C.G. tumour vaccines and to apply the resulting knowledge and experience to the treatment of patients.

Studies with guinea pigs ^(226, 227), mice ^(228, 229) and rats ^(230, 231) have demonstrated complete suppression of tumour growth when tumour cells were mixed with B.C.G. before injection into syngeneic recipients, whereas injection of B.C.G. and tumour cells at separate sites did not affect the growth of the tumour.

Effective immunotherapy of established leukaemia, lymphoma, and sarcoma transplants ^(232, 233) has been observed in mice and rats after the administration of B.C.G. alone or in combination with irradiated, viable or neuraminidase-treated tumour cells.

The growth of methylcholanthrene induced sarcoma was controlled or suppressed by B.C.G. given intravenously ⁽²³⁴⁾ in rats and attempts at additional immune stimulation by the addition of irradiated tumour cells mixed with B.C.G. failed to provide more effective control of post-excision pulmonary metastases.

Similarly, in a rat epithelioma system additional immunostimulation with irradiated Spl tumour cells mixed with B.C.G. did not provide more effective control of pulmonary metastases than intravenous B.C.G. alone ⁽²³⁵⁾.

In the hepatocarcinoma model in guinea pigs, a vaccine containing live tumour cells and B.C.G. was effective in treating animals with an intradermal tumour burden of 10^5 to 10^6 cells but not for animals with 10^7 cells ^(236, 237).

Comparison of the effect of a single dose (0.015 mgm/g) B.C.G. administered by different routes, on the growth of Bl6 mouse melanoma, showed that if B.C.G. was given 10 days before tumour challenge a significant resistance to tumour growth was observed regardless of the site of tumour challenge or the route of B.C.G. administration. The effect was less marked if the B.C.G. was given after tumour implantation ⁽²³⁸⁾.

Growth of intrapleurally injected cells of immunogenic methylcholanthrene induced sarcomas and of an ascitic hepatoma was suppressed by intrapleural injection of viable or radiation sterilised B.C.G. vaccine ⁽²³⁹⁾. 200 or 500 μ g

moist weight of B.C.G. (Glaxo strain) containing 3×10^8 viable organism per ml was effective given several days before or after tumour challenge.

B.C.G. injected intravenously, subcutaneously or intraperitoneally was without influence on pleural tumour growths. I.P. growth of these tumours was suppressed only by I.P. injection of B.C.G. In contrast no effect could be demonstrated using two other tumours which lacked significant immunogenicity.

In patients attempts to control the progression of established tumours by B.C.G. alone or B.C.G. tumour cell vaccines have been less successful than might have been hoped for in light of the results from animal experiments.

B.C.G. given by scarification to four patients with metastatic malignant melanoma resulted in progression of the disease in three patients and one patient responding over a period of 2.5 years⁽²⁴⁰⁾. In another series of ten patients with metastatic malignant melanoma⁽²⁴¹⁾, B.C.G. by scarification resulted in no conclusive evidence that the treatment was either beneficial or harmful. Treatment of seven patients with disseminated malignant melanoma by oral ingestion of B.C.G. resulted in the lesions of three patients regressing, two progressing and no detectable change in the lesions of the other two⁽²⁴²⁾.

In one study B.C.G. (0.8×10^6 viable bacilli, tice strain, in 0.1 ml) given by intradermal injection appeared to delay the progression of malignant lymphoma⁽²⁴³⁾. In the control series 77% relapsed, within the follow-up period of 6 - 8 years, with a mean time to first new lesions of 10.6 months. Of the vaccinated patients 61% relapsed, with a mean time to first new lesions

of 25.9 months. In this study, however, the vaccinated patients were in remission longer than the un-vaccinated at the time of inclusion in the trial, and in general in malignant lymphomas the longer the remission the less likelihood of relapse. A larger proportion of the patients vaccinated had stage I disease (as opposed to stage II, in the un-vaccinated), a larger proportion were women, and a larger proportion had a longer duration of disease before treatment, all of which are favourable prognostic factors.

Twenty-one patients with Philadelphia positive chronic myeloid leukaemia treated with B.C.G. and cultured cells developed persistent delayed hypersensitivity to antigens of the target cells and the clinical impression was of benefit ^(244, 245). However, this study was uncontrolled, vaccination schedules were variable, and the cultured cells were later found to be lymphoid lines. The authors noted that the results were compatible with non-specific immunostimulation since the presence of tumour specific antigen in the vaccine could not be proved.

Four of eleven patients, with surgically incurable malignant melanoma, without skin metastases, treated by intradermal injections of a vaccine containing irradiated autochthonous tumour cells and B.C.G. ⁽²⁴⁶⁾ had objective responses. Remission was associated with a modest tumour burden confined to subcutaneous and lymph node metastases.

However, no definite conclusions can yet be drawn from the data so far available as to the efficacy of either non-specific B.C.G. or B.C.G. - tumour vaccine therapy in the treatment of established tumours due to the small numbers involved and to the bad experimental design in some cases.

The tumour load appears to constitute a limiting factor in immunotherapy. Systemic administration of B.C.G. and irradiated leukaemic cells could only cure mice if the tumour load did not exceed 10^5 cells⁽²³²⁾. Intralesional injection of B.C.G.⁽²³⁷⁾ induced complete regression in 60% of guinea pigs with intradermal hepatoma transplants weighing 95 mg, but in less than 20% with tumours weighing 425 mg.

In view of the evidence that the number of tumour cells is of importance in determining the immune system's ability to reject tumours the reduction of the tumour burden may allow B.C.G.'s augmentation of immunity to eliminate the remaining tumour cells. In mice s.c. inoculation of 1×10^4 L.S.T.R.A. lymphoid leukaemia cells led to detectable systemic leukaemia in 7 days and death in 14 to 17 days. A single treatment of B.C.N.U. prolonged life span 20 - 25% ; the addition of B.C.G. increased survival with 50 to 100% of animals being 'cured' of leukaemia⁽²⁴⁷⁾. Best results were obtained if the B.C.G. was administered with minimal tumour burdens.

In contrast no favourable effect could be demonstrated in the N.O.V.A. rat leukaemia treated with B.C.G., after cyclophosphamide to reduce the tumour burden, but it should be noted that in the N.O.V.A. leukaemia no tumour associated Ag has been demonstrated⁽²⁴⁸⁾.

In syngeneic mice given Gross virus induced leukaemia cells, the addition of B.C.G., i.p., after cyclophosphamide tumour reduction, failed to prolong survival⁽²⁴⁹⁾.

After surgical reduction with incomplete excision of a rat polyoma tumour, treatment with B.C.G. increased the growth of remaining tumour with

an associated increase in serum blocking activity⁽²²⁵⁾, although if associated with previous splenectomy and inoculation of unblocking serum, B.C.G. led to tumour regression.

In a tumour system (mammary adenocarcinoma implantation) in which surgical excision of the growing tumour alone resulted in 100% mortality from spontaneous metastases, preoperative injection of B.C.G. into the tumour or post operative therapy with B.C.G. alone or B.C.G. - tumour cell vaccine resulted in significantly prolonged survival and the possibility of 'cure' since no residual tumour could be found at the termination of the experiment in these animals⁽²⁵⁰⁾.

In patients with a variety of carcinomas the use of a B.C.G. vaccination associated with alkylating agents appeared to show significant survival increase at 6 months, 12 months and 24 months⁽²⁵¹⁾. Unfortunately this study contains more cases of breast cancer in the B.C.G. treatment groups and if the tumour was known to be hormonally responsive then hormones were given ; with the alkylating agent a 'leucocyte boosting thymus extract' was given ; more members of the treatment groups received alkylating agents ; objective responses were not recorded ; a portion of the control group was retrospective and the dose of B.C.G. in terms of living organisms is unknown.

In 20 of 35 patients with carcinoma of head and neck, treated with intradermal B.C.G., isoniazid and methotrexate, there was a reduction in gross tumour volume lasting more than 60 days⁽²⁵²⁾. There is, however, no conclusive evidence in this uncontrolled study that B.C.G. and isoniazid added anything to the results obtained with methotrexate alone.

24 of 32 patients with Burkitt's disease, with remission after cyclophosphamide therapy, were randomised into B.C.G. and control groups (253). No protective or harmful effects of B.C.G. immunotherapy in relation to relapse frequency or duration of remission were noted.

A highly significant difference has been reported between two groups of patients with acute lymphoblastic leukaemia, given B.C.G. by scarification, after tumour load reduction by systemic chemotherapy, sequential complementary systemic chemotherapy and intrathecal chemotherapy or irradiation. Patients without immunotherapy relapsed after an average remission of 66 days; of the patients with immunotherapy, either B.C.G. (Pasteur strain) or formaldehyde inactivated allogeneic cells only 9 from 20 had relapsed at 130 days (254, 255, 256, 257).

In acute myelogenous leukaemia, after remission induction by a 5 day course of daunamycin and cytosine arabinoside, tumour vaccine was used in the maintenance of remission (258). The patients were divided into three groups; the first receiving chemotherapy alone, the second receiving the same chemotherapy plus Glaxo B.C.G. (1×10^6 live organisms delivered by the heaf gun) and 1×10^9 irradiated leukaemic cells administered s.c. and i.d., the third receiving the same form of immunotherapy alone. The groups receiving immunotherapy alone, and immunotherapy plus chemotherapy did significantly better than the group receiving chemotherapy alone. Whether the B.C.G., the irradiated cells or the combination were active in prolonging the remission is uncertain.

Remission after chemotherapy was significantly prolonged in acute myelocytic leukaemia, by B.C.G, immunotherapy, using Tice B.C.G. in combination with methotrexate, compared with methotrexate alone ⁽²⁵⁹⁾.

B.C.G. (Tice) induced significant improvement in survival in patients with surgically resected recurrent malignant melanoma compared, however, to surgery alone in retrospective controls ⁽²⁶⁰⁾.

Comparison of remission duration of malignant melanoma and cellular reactivity to both primary and recall antigens, between Glaxo B.C.G. given i.d., and Pasteur Institute B.C.G. given by scarification, suggested that the Pasteur B.C.G. by scarification was associated with an increased duration of remission and increased cellular reactivity ⁽²⁶¹⁾. Unfortunately, the the validity of the comparison, the Pasteur dose was 1×10^4 times greater than the Glaxo dose.

In a contrasting study in acute lymphatic leukaemia, using a different strain of B.C.G. (Research Foundation, Chicago), and without central nervous system irradiation, the B.C.G. treated group did poorly as compared to the other 2 groups ⁽²⁶²⁾. After remission induction with prednisolone and vincristine followed by methotrexate I.V., 26% of patients remained in remission on no therapy, 21% in remission on B.C.G. therapy and 82% stayed in remission on maintenance therapy with prednisolone, vincristine and methotrexate.

191 cases of acute lymphoblastic leukaemia treated with prednisolone, vincristine, β -mercaptapurine, L-asparaginase and high dose methotrexate and folinic acid rescue, were randomised into 3 groups: no further treatment, twice weekly methotrexate, and B.C.G. immunotherapy. Remission length

was greatest with methotrexate (52 weeks), intermediate with B.C.G., (27 weeks), and worst with no treatment (17 weeks) ⁽²⁶³⁾. In this trial the B.C.G. used was Glaxo freeze dried and apart from causing lymphocytosis had no other conspicuous effect.

As an adjunct to chemotherapy, vincristine and D.T.I.C., in patients with disseminated malignant melanoma, an immunisation protocol of the i.d. inoculation of 2×10^7 irradiated allogeneic melanoma cells, admixed with 50 µg of B.C.G., resulted in a high number of objective regressions, associated with a transient fall in specific inhibitory effects of the patient's sera, on tumour directed cytotoxic activity, of the patient's lymphocytes ⁽²⁶⁴⁾. This is, however, a preliminary report without concurrent controls.

In vitro studies showing that ascites tumours and lymphomas could be destroyed by the close approximation of macrophages stimulated by B.C.G., and the use of intra-lesional B.C.G. to induce tumour regression of a carcinogen-induced transplantable hepato-carcinoma in Sewall-Wright guinea pigs stimulated interest in this method as an immuno-therapeutic approach to cancer ^(187, 226). The results obtained indicate that the growth of i.d. transplanted syngeneic tumours can be suppressed by intra-lesional B.C.G., not due to a direct cytotoxic effect of B.C.G. on the tumour cells, but requiring the participation of the hosts immune defences ⁽²⁰⁰⁾. Optimal tumour suppression requires B.C.G. in close contact with the tumour cells.

Suppression of tumour growth in skin is more effective than suppression of tumour growth in draining lymph nodes and this limitation of the effectiveness

of intra-lesional B.C.G. is also apparent for intra-muscular tumour implants ⁽²²⁰⁾. In contrast B.C.G., Phipps strain, injected into established colon wall tumours of transplanted line - 16 hepato-carcinoma in guinea pigs, suppressed tumour growth if the tumour burden was limited ⁽²⁶⁵⁾, emphasising the possibility of immunotherapy in other than superficial sites.

In mice i.d. injection of B.C.G. into a methylcholanthrene induced sarcoma prevented progressive growth of the sarcoma ⁽²²⁸⁾, the suppression being dependent on the number of organisms injected, 10^7 colony forming units (C.F.U.) suppressing the growth of 5×10^5 tumour cells in 100% mice, whereas 10^6 C.F.U. suppressed the growth of 5×10^5 mice in 50% mice. The use of heat killed B.C.G., or local inhibition of B.C.G. multiplication with isoniazid, did not interfere with local tumour suppression, but the elimination of immunologically committed small lymphocytes with mouse anti-thymocyte serum or cortisone acetate did abolish B.C.G. suppression of tumour growth, in this model.

In a murine system employing three tumours, two of which were demonstrated to be of low antigenicity, tumour cells failed to grow when injected into syngeneic animals as a mixture with B.C.G. ⁽²⁶⁶⁾. This effect was limited to tumour cells given at the same site as B.C.G. In non-immune immunosuppressed mice the administration of B.C.G. - tumour mixture was associated with a temporary growth delay suggesting non immunological processes only partially suppress tumour growth.

A weakly immunogenic rat epithelioma of spontaneous origin, when implanted s.c. with B.C.G. showed inhibition of local tumour development, decreased pulmonary metastases and increased animal survival ⁽²³⁵⁾. Similar results occurred when B.C.G. was given with rapidly growing methycholanthrene- induced sarcoma cells in rats ⁽²³⁴⁾. Rejection of the tumour - B.C.G. inoculation resulted in suppression of growth of a simultaneous challenge with cells of the same tumour at a contralateral s.c. site.

Of seven patients treated with intra-lesional B.C.G., four responded and two failed to respond. Assessment of the patients indicated that response was associated with an increased immune reactivity, as measured by various parameters, (lymphocyte stimulation by saline extract of melanoma or P.H.A. inhibition of leukocyte migration to melanoma Ag, an increased T cell lymphocyte population as demonstrated by rosette formation with S.R.B.C., in vitro lymphocytotoxicity against melanoma lines, or formation of anti-melanoma Ab), and the presence of disease limited to the skin and lymph nodes ⁽²⁶⁷⁾.

Weekly intranodular injections of B.C.G. (lyophilised Pasteur B.C.G.) into secondary cutaneous and subcutaneous melanoma deposits, until suppuration occurred, gave no indication of even a slight clinical or histological effect on the uninfected control nodules ⁽²⁶⁸⁾. Although no recurrence occurred in the 485 injected nodules only 2 of the 25 patients had no recurrent clinical tumour 2 years after completion of local treatment.

12 patients with advanced malignant melanoma received 0.1 ml B.C.G. into multiple nodules on one occasion. Five of the 12 had temporary regression of the injected nodules and 2 of this group had regression of uninjected nodules, and one of these was tumour free at 3 years follow up ^(269, 270). The regressions were associated with sensitivity to D.N.C.B. and increase in anti-tumour antibodies. The patient with the complete sustained regression was the only one to have only skin lesions and no lymph node or visceral disease.

An extension of these studies showed that of patients with skin metastases of malignant melanoma only, direct injection of the skin metastases with B.C.G. resulted in 90% regression of injected lesions, 17% regression of uninjected lesions in immunocompetent patients ⁽²⁷¹⁾, and approximately 25% of patients remained free of diseases for 1 - 6 years. A lower incidence of local control and no long term survivors were the results in patients with subcutaneous or visceral metastases.

B.C.G. and prevention of tumour development.

Experimentally B.C.G. has afforded protection against subsequent challenge with a variety of tumours, by decreasing the tumour incidence, delaying the development of tumours and increasing the survival of the hosts ^(7, 162, 163, 272, 273). The effects were demonstrated in various small animals, and against tumours administered by a variety of routes.

Favourable effects of B.C.G. could also be demonstrated against oncogenesis by a variety of viruses ^(273, 274, 275), and B.C.G. combined with specific tumours, also afforded protection against subsequent tumour challenge ^(276, 236, 266, 232).

In patients analysis of the subsequent development of neoplasia in groups of individuals immunised with B.C.G. gives contradictory results often due to poor experimental design and statistical interpretation, and failure to appreciate the varying reactions of B.C.G. in different populations ⁽¹⁵⁴⁾.

The original analysis of data from Quebec ⁽²⁷⁷⁾ found death from leukaemia to be less common in B.C.G. vaccinated individuals. Objections to the method of statistical analysis ⁽²⁷⁸⁾ lead to reappraisal of the results and re-analysis suggested no relationship between B.C.G. immunisation and the incidence of leukaemia ⁽²⁷⁹⁾. Similar results and interpretation to these latter figures were produced for Glasgow ⁽²⁸⁰⁾.

A study from Chicago ⁽²⁸¹⁾ suggested a significant decrease in leukaemia mortality in B.C.G. immunised children. However, this study was retrospective and not randomised so that a definite demographic group was selected.

A controlled randomised trial in Georgia and Alabama revealed no difference in the subsequent development of leukaemia, lymphosarcoma or Hodgkin's disease in B.C.G. immunised children ⁽²⁸²⁾. It should, however, be noted that in this study the B.C.G. immunisation programme gave no increased protection against subsequent tuberculosis ⁽²⁸³⁾. This emphasises that the variability in the protection afforded by B.C.G. against tuberculosis is likely to be reflected in a similar variability in effectivity against tumours and cause similar problems in the interpretation of the results.

In a controlled trial in Puerto Rico, with an average follow up of 18.8 years, it was found that B.C.G. immunised children had a very slightly decreased incidence of leukaemia, but an excess of lymphosarcoma and Hodgkin's disease ⁽²⁸⁴⁾.

On the prevention of tumours induced by chemical carcinogens the effect of B.C.G. has been much less conclusive than in other experimental models and since this is the aspect most related to the experimental work of this thesis the relevant experiments are given in detail.

B.C.G. and chemical carcinogenesis.

3-methyl cholanthrene dissolved in sesame oil was injected into the right thigh muscle of Swiss 11 A. / I.C.R. mice. B.C.G. infection (1 mgm wet weight) prior to carcinogen injection did not consistently alter the rate of tumour appearance or the final incidence. Infection with B.C.G. 86 days following carcinogen injection caused a significant delay in the appearance of tumours observed although the final cumulative incidence was similar to that seen in non-infected animals ⁽⁸⁾.

Subcutaneous injection of 0.5 mg M.C.A. dissolved in 0.1 ml olive oil produced tumours in 80% of control S.W.M./M.S. control mice. Intradermal injection of 0.1 ml B.C.G. suspension containing 5×10^7 viable cells 2 weeks before M.C.A. administration decreased tumour incidence significantly. B.C.G. at the time the first mice were detected having palpable tumours was without effect in reducing tumour incidence ⁽²⁸⁵⁾. However, it should be noted that the early injections were given i.d. and the latter group i.p., and that in the groups receiving i.p. injections the tumour incidence was slightly greater than the controls.

B.C.G., 1 mg living Bacilli given s.c., on the same day as D.M.B.A. delayed appearance of mammary carcinomas in rats although the ultimate

incidence of tumours was similar. B.C.G. given before, or within six weeks after, the D.M.B.A. feeding had no effect on the rate of tumour production and B.C.G. was given after the appearance of the first tumour, accelerated subsequent tumour growth ⁽²⁸⁶⁾. In these experiments B.C.G. appeared to counteract the effects of D.M.B.A. induced depression of the spleen weight and the reduction of mononuclear spleen cells.

In C57BL/6 mice 1 ml B.C.G. administered I.P. (5×10^7 organisms / ml), given either 2 weeks before or 2 weeks after carcinogen administration, 500 µg 3 M.C.A., provided significant temporary protection as demonstrated by a decreased rate of tumour appearance ⁽²⁸⁷⁾. Methanol extracted residue at a dose of 0.5 mg provided relatively little protection, but at a dose of 2.5 mg provided significant temporary protection. In these experiments, however, with the amount of 3 M.C.A. used, (500 µg I.M.), almost all the mice developed tumours.

The hamster cheek pouch, using D.M.B.A. as the carcinogen provides a useful model since the tumours apparently develop in recognisable sequential manner, the epidermoid carcinomas following the formation of hyperkeratosis and dyskeratosis. Using this model, pouches from control hamsters painted with D.M.B.A. had uniform well developed epidermoid carcinomas when examined histologically at 12 weeks. In contrast hamsters receiving B.C.G. (1 mg B.C.G. x 2 weekly for 14 weeks, starting week - 2 relative to painting with D.M.B.A.) had hyperkeratosis, some dyskeratosis and rare foci of epidermoid carcinoma ⁽²⁸⁸⁾. The changes at 12 weeks in the B.C.G. group

resembled those in a group of controls that had been killed at 8 weeks, suggesting significant delay in the progression of carcinogenesis by B.C.G.

Charles River mice, painted once with 125 µg D.M.B.A. (tumour initiation) followed by repeated applications of croton oil, 0.6 mg twice weekly for 25 weeks (tumour promotion) received I.V. 1.0 mg B.C.G. in 0.1 ml. 0.08% NaCl, 2 days after the D.M.B.A. application⁽²⁸⁹⁾. This reduced the rate of appearance and incidence of both benign and malignant skin tumours. In these experiments the animals treated with B.C.G. which developed tumours had a larger spleen index than those animals which did not develop tumours. This may be due to the inability of the tumour bearing animals to destroy the tumours although they have the ability to recognise them. Thus, the reticulo-endothelial system is continually stimulated by the tumour antigens which it cannot eliminate. This failure of elimination may be due to the local immunosuppressive effect on the long continued croton oil applications or it may be that the enlarged spleen is producing antibody which, complexed to antigen, is blocking any cell mediated cytotoxic activity.

Persistent generalised B.C.G. infection, following the intra-colonic injection of B.C.G. (6.7×10^6 organisms) in rats, had no effect on the rate of development or incidence of tumours induced by 1,2 - dimethylhydrazine dihydrochloride (D.M.H.) given intragastrically for 5 weeks, but did increase the number of mucinous adenocarcinomas and metastases⁽²⁹⁰⁾.

Part IV

PRELIMINARY EXPERIMENTS :

- (I) TO CORRELATE THE CYTOLOGICAL CHANGES, THE MACROSCOPIC APPEARANCES AND THE HISTOPATHOLOGY.
- (II) TO DEMONSTRATE THE IMMUNOGENICITY OF THE TUMOURS PRODUCED BY THIS METHOD.

(I) To ensure as far as possible that the cytological picture accurately reflected the progression of the neoplastic changes, preliminary experiments were carried out to determine which cytological features correlated with the stage of carcinogenesis as determined by the histological examination.

After the carcinogen implantation, the mice were followed by weekly cytological examination and immediately after the cytological examination each week, a batch of five mice was killed. The macroscopic and histological features of the cervix were correlated with the cytological appearance of the exfoliated cells.

Pathological Anatomy

In those cases in which no tumour occurred the cervix either appeared little different from normal apart from slight thickening or had a rough granular surface, but without any ulceration. Both of these appearances could be associated with intra-epithelial malignant lesions, the rough granular surface being associated more commonly with the more severely dysplastic type of lesion. These appearances were found also associated with the formation of cords or strands of cells apparently invading into the underlying stroma or with definite micro-invasion.

The macroscopic appearances associated with the first cytological evidence of invasion were of thickening of the tissue around the cervical canal, giving a tubular, slightly swollen appearance. The epithelial surfaces appeared either granular or papillary and bulged into the lumen of the canal.

The majority of the invasive carcinomas were of the exophytic type and varied considerably in size. The lesions were located at the external os or in the endocervical canal. The smaller lesions were papillary and filled the endocervical canal; the larger tumours were often ulcerated and necrotic and invaded down into the vagina and up into the uterine horns.

Invasion into and around the rectum occurred and the ureters were obstructed by surrounding tumour masses.

Metastases occurred in many cases, mainly to the lymph nodes of the lumbar region.

Histopathology

The initial response of the cervical tissue to the 20 M.C. implantation was inflammatory with the formation of small micro-abscesses, containing polymorphonuclear leukocytes, in the epithelium. In the underlying connective tissue there was focal oedema also associated with collections of polymorphonuclear leukocytes. After the initial inflammatory response, lasting about two weeks, the epithelial cells showed slight cytological variation with cell crowding, slight nuclear enlargement and hyperchromasia, but no increase in mitotic figures. As the lesions progressed, disordered proliferation of the basal cells gave rise to a compact very cellular stratum replacing the normal

single layer (Fig. I). Associated with this there were cellular atypias and some increased mitotic activity. The degree of atypia, however, was not marked. The orderly differentiation of the epithelium was also disturbed and a diffuse polymorphonuclear cell infiltrate was present throughout the epithelium. The epithelial lesions were usually diffuse, but occasionally isolated segments of sharply demarcated, increased basal activity could be demonstrated (Fig. II). The cells in these segments contained enlarged, densely clustered nuclei, and were often sharply demarcated from the upper epithelial layers. These changes suggested the beginning of epithelial transformation with the growth of a new cell type, while the more diffuse proliferation associated with the polymorphonuclear infiltration suggested reaction to the irritant properties of the carcinogen.

With further progression more advanced atypical basal cell hyperplasia could be found with the basal cells showing enlargement and pleomorphism of the nuclei which were crowded together (Fig. III). Mitoses were increased and in the enlarged nuclei, nucleoli were prominent. Above the atypical basal cell layers the epithelium was sharply demarcated and showed little abnormality.

Advanced atypical basal cell hyperplasia was often associated with two intra-epithelial lesions, (1) Dysplasia (2) In-situ carcinoma.

In moderate dysplasia above the atypical basal cells the number of nuclei decreased rapidly, but remained atypical throughout. Mitoses occurred only in the basal layers and leukocyte infiltration was found in the middle layers. There was an abnormally thickened and abnormally differentiated

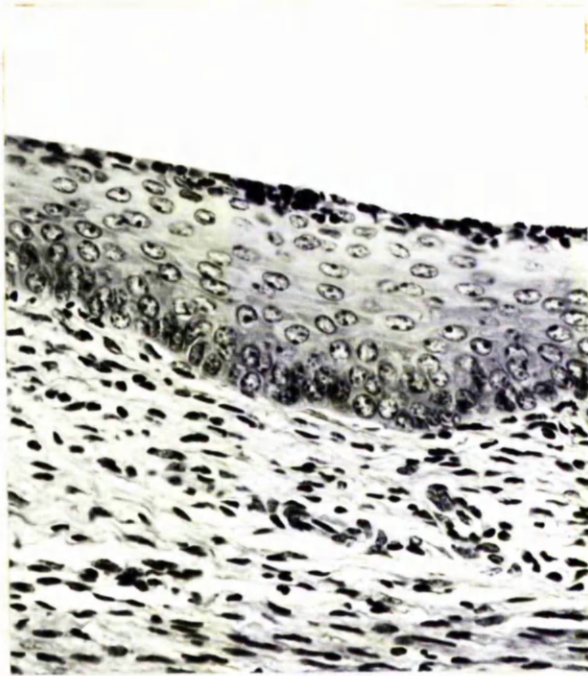


FIG. I. Basal cell hyperplasia : replacement of single basal cell layer by several strata of cells.

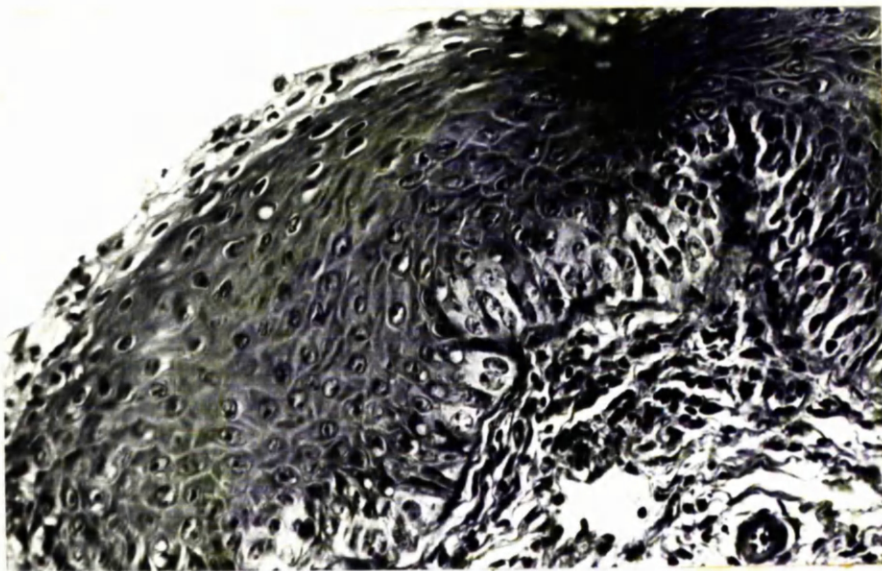


FIG. II. Short segment of atypical hyperplastic basal cells suggestive of epithelial transformation to a new cell type.

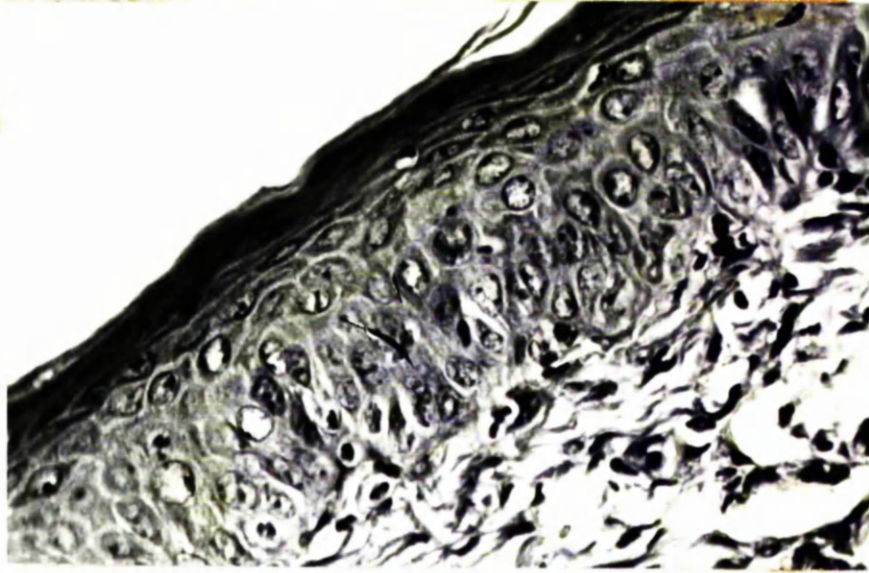


FIG. III. More advanced atypical basal cell hyperplasia with pleomorphic cells and nuclei.

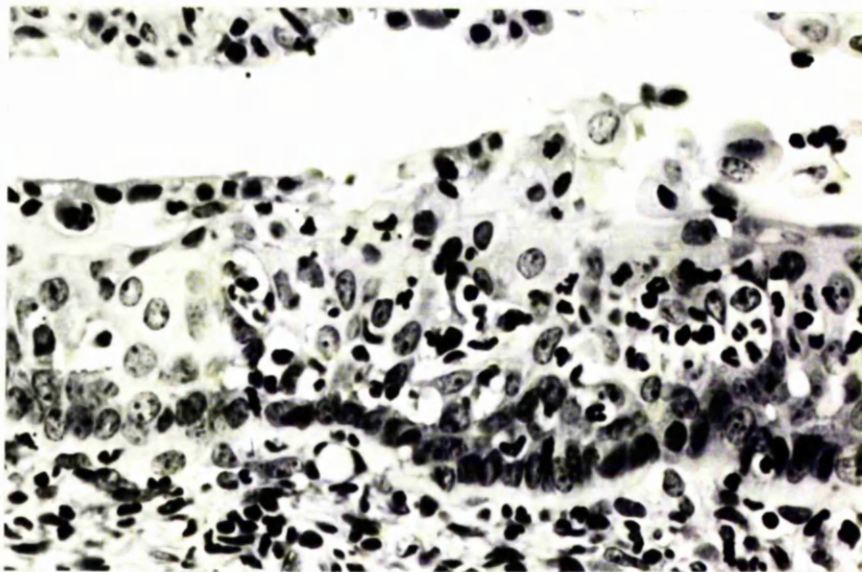


FIG. IV. Moderate dysplasia with atypical nuclei through the full thickness of the epithelium.

basal cell layer (Fig. IV). On the surface there was pyknosis and parakeratosis. Occasionally associated with moderate dysplasia there were bulky epithelial outgrowths into the underlying stroma, but the basement membrane areas associated with these remained intact.

In severe dysplasia the architecture of the epithelium was disordered in the basal layers, and this was associated with marked nuclear pleomorphism and atypia (Fig. V). The cells however, in all layers remained clearly defined. Mitotic activity was greatly increased but was limited to the basal layers. In all cases some degree of differentiation took place in the upper layers. Severe dysplasia was often associated with bulky downgrowths of epithelium into the connective tissue stroma or with slender pegs of epithelium separated by elongated stromal papillae. In some areas foci of nuclear clumping could be found.

In carcinoma in situ the normal stratified structure of the epithelium was no longer recognisable due to the great increase in atypical cells and nuclei (Fig. VI), through the entire thickness of the epithelium. Cell borders were difficult to recognise. The epithelial structure was severely disordered because of the complete loss of nuclear polarity and in many cases mitoses were present right up to the surface. The cells forming the carcinoma in-situ were often spindle-shaped undifferentiated cells although others had larger nuclei and more variations in nuclear form. Bulky outgrowths of the epithelium into the underlying stroma often occurred, and in these outgrowths the cells often showed numerous mitoses, but the basal cell layer always remained well defined and bordered the intervening stromal papillae.

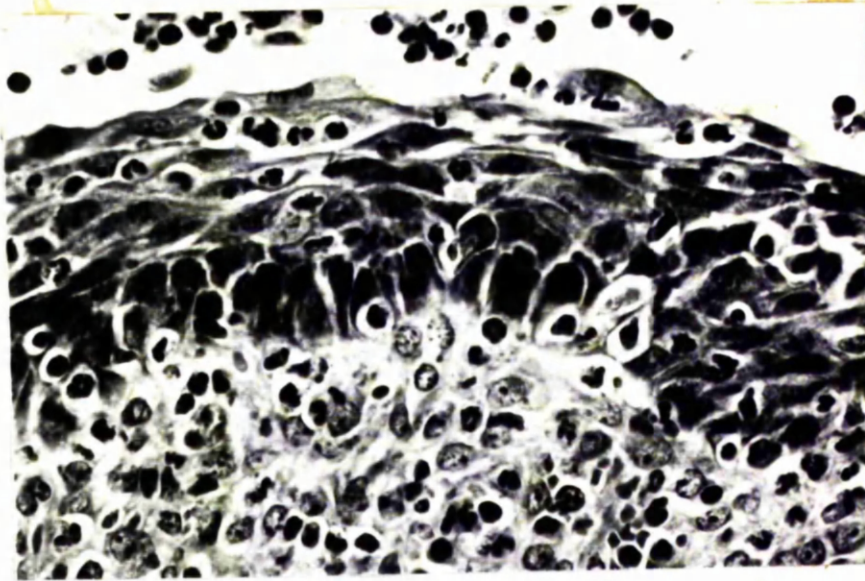


FIG. V. Severe dysplasia with disordered architecture associated with marked nuclear pleomorphism and atypia.

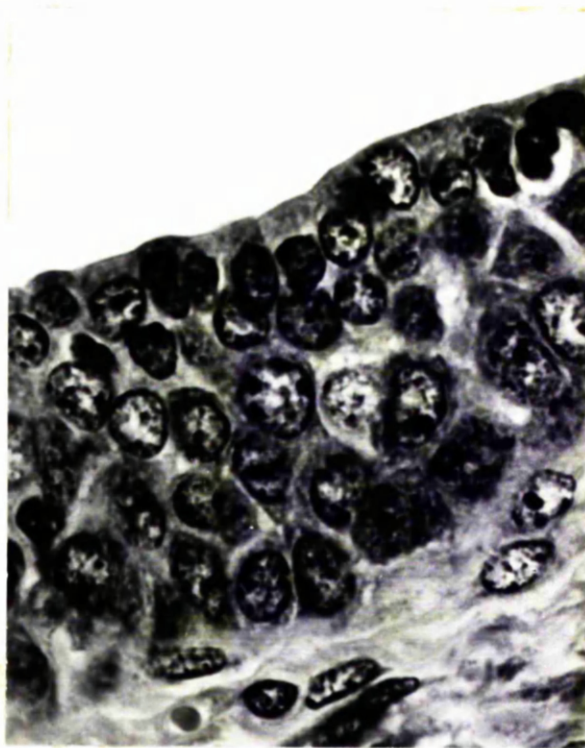


FIG. VI. Carcinoma in-situ with greatly increased numbers of atypical cells and nuclei resulting in loss of normal stratified structure.

Early invasive growth was always found in association with an atypical epithelium, either from severely dysplastic epithelium or from in-situ carcinoma (Fig. VII). The majority of the invasive lesions were associated with severe dysplasia and the minority with in-situ carcinoma. Only one case of invasive carcinoma was found associated with a relatively mild degree of dysplasia.

In some cases one change suggestive of invasion was the appearance of a dense lymphoid and plasma cell infiltrate in the stroma beneath the epithelium (Fig. VIII). The focal inflammatory response was so dense that the border between the epithelium and stroma could not be identified.

Pointed and forked invasive pegs often grew down from either dysplastic epithelium or in-situ carcinoma (Fig. IX). The pegs often showed more differentiation than the epithelium from which they were apparently arising, and they were not surrounded by any recognisable basal membrane layer.

Thin cords of atypical basal cells could also be found penetrating deeply into the stroma often eliciting little evidence of any tissue response (Fig. X).

The progression of the micro-invasive lesions took the form of advancing cords of epithelial cells into the stroma with numerous branching processes (Fig. XI). In the immediate neighbourhood of the invasive lesion there was stromal oedema and a lymphoid cell and macrophage infiltrate.

In other lesions the invasion was on a broad front over a segment of epithelium with relatively little evidence of stromal changes or an inflammatory response, and similar type of invasion appeared to arise from nodular epithelial downgrowths (Fig. XII).

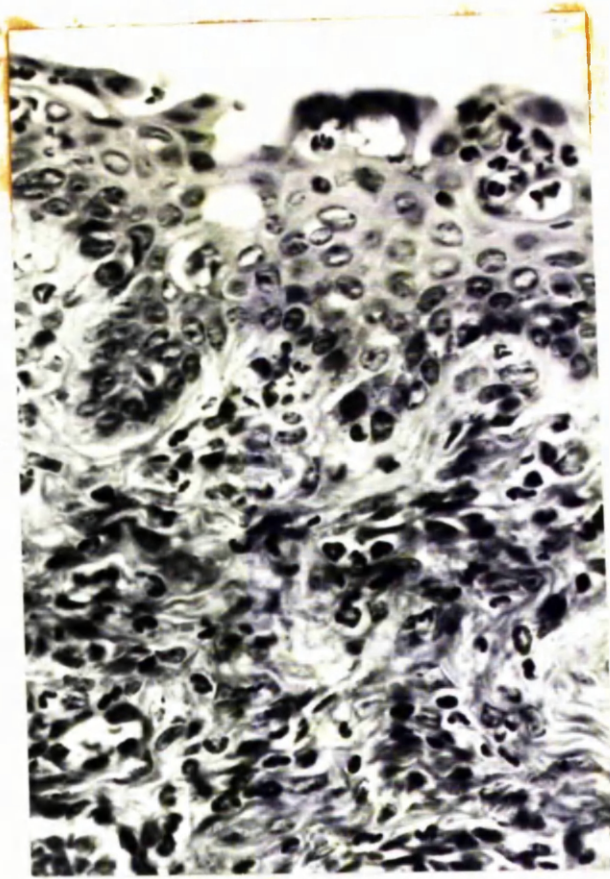


FIG. VII. Early invasion from a dysplastic epithelium.

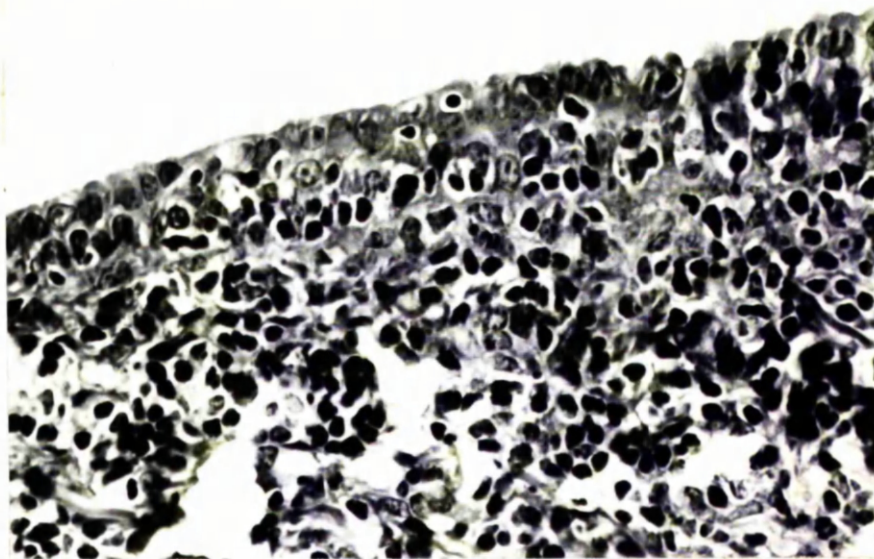


FIG. VIII. Early invasion with associated lymphoid and plasma cell infiltrate in the underlying stroma.

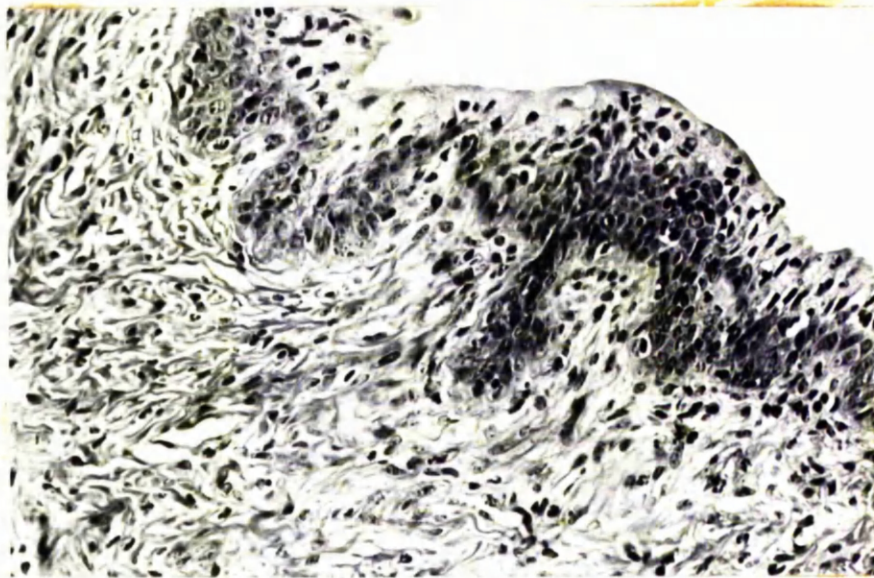


FIG. IX. Invasive pegs growing down from dysplastic epithelium.

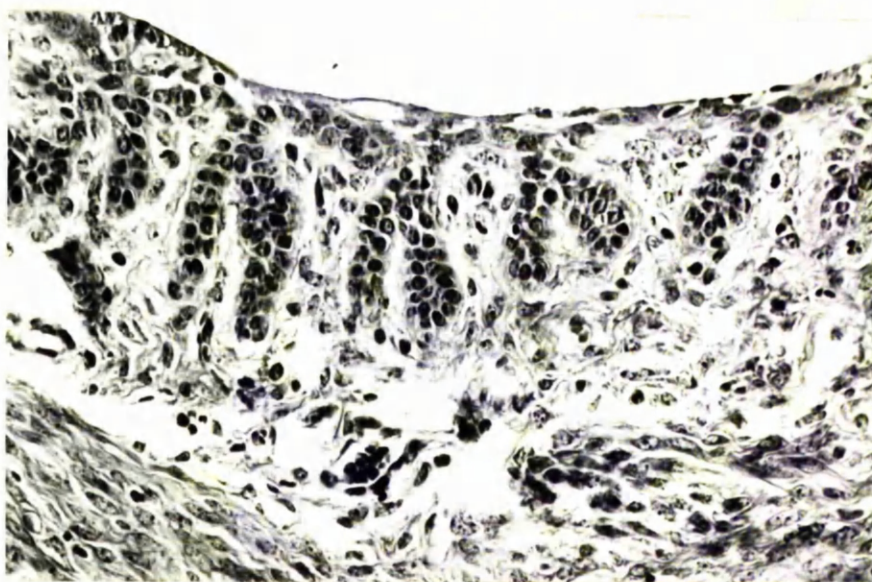


FIG. X. Cords of atypical basal cells penetrating into the stroma with little tissue response.

The progression of the invasion ultimately gave rise to the tumours of cervix, the majority of which were well-differentiated squamous carcinomas (Fig. XIII), but a few moderately well-differentiated carcinomas were also produced. These tumours ultimately invaded locally to the bladder, around the ureters and into the wall of the rectum (Fig. XIV). Metastases to local lymph nodes also occurred.

When the invasive lesion was well advanced the lymphoid infiltrate was limited to the periphery and often was markedly reduced. At this stage it consisted mainly of small lymphocytes and scattered plasma cells.

Cytopathology

The early response of the cervical epithelium to the implantation of 20 M.C. was reflected in the vaginal smear by large numbers of polymorphonuclear leukocytes, many clumped around necrotic squamous cells, correlating with the tissue sections showing focal polymorphonuclear infiltration with the formation of small micro-abscesses in the epithelium. This inflammatory exudate continued for two weeks and then subsided.

After the initial inflammatory response the exfoliated cells were characterised by increasing numbers of basal and parabasal cells in which there were cytoplasmic alterations (Fig. XV). These consisted of increased cytoplasmic basophilia, some cells having fine granularity, others being very coarse-grained. This cytological picture and cell type corresponded with the early dysplastic changes in the cervical epithelium.

As the lesions advanced, plump hyperchromatic basal cells made their appearance in large numbers (Fig. XVI). These cells corresponded to the cervical changes of severe dysplasia, in which there was disordered growth

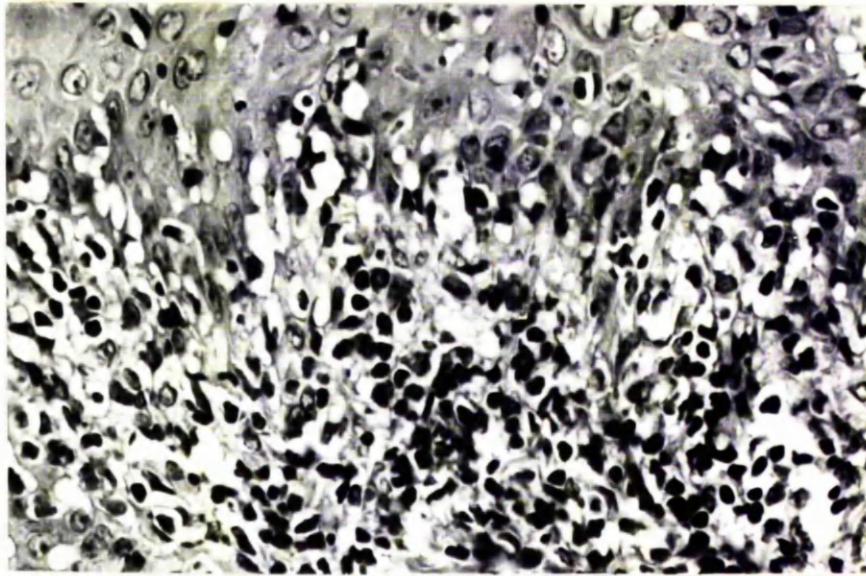


FIG. XI. Epithelial cells invading into the stroma with branching processes ; there is associated stromal oedema and a lymphoid infiltrate.

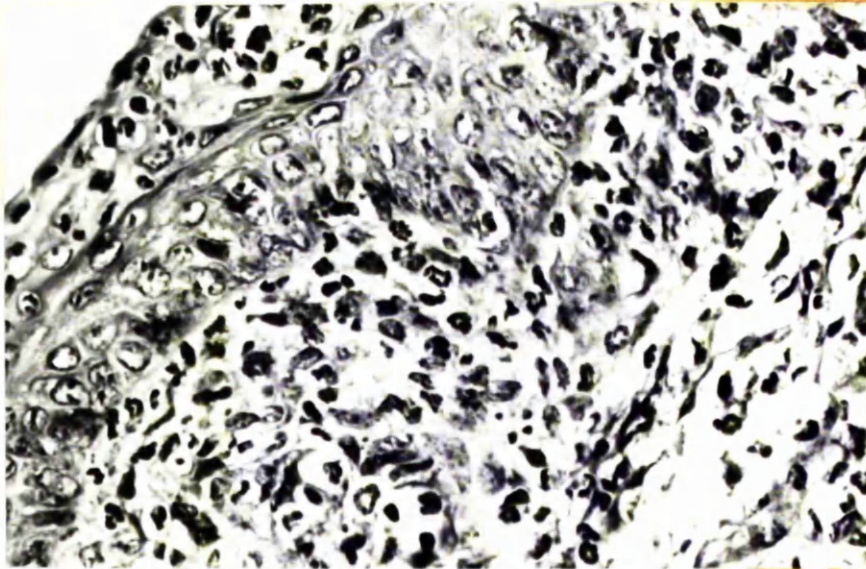


FIG. XII. Invasion arising from a nodular epithelial downgrowth.

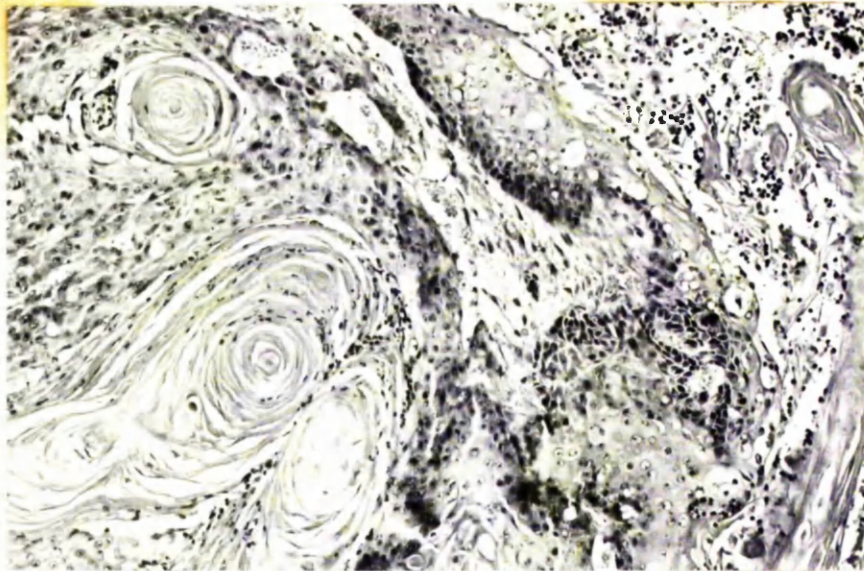


FIG. XIII. Advanced squamous carcinoma of cervix.

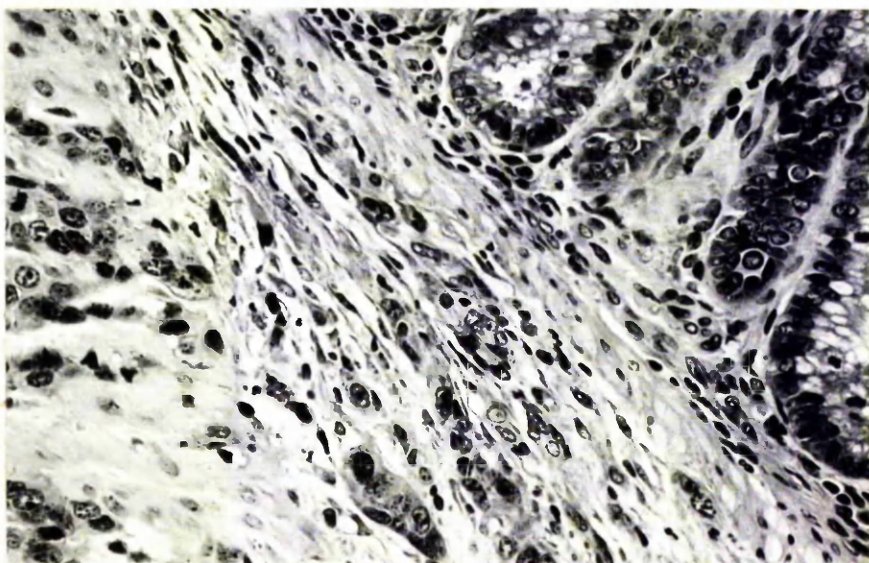


FIG. XIV. Squamous carcinoma invading into the wall of the rectum.

and increased mitotic activity of the basal and parabasal regions and the presence of plump nucleated parabasal cells near the surface. The surface was formed by a thin layer of keratin. Later many of the exfoliated cells showed nuclear evidence of malignant transformation similar to that of the cells exfoliated in in-situ carcinoma. It was these cells, together with those found associated with in-situ carcinoma, that were regarded as the first malignant cells (Fig. XVII).

In the animals showing the lesion consistent with carcinoma in-situ the exfoliated cells showed marked variation in shape and size with large nuclei showing a definite increase in the nucleo-cytoplasmic ratio (Fig. XVIII). Multinucleate cells were also present (Fig. XIX). The chromatin pattern was coarse and the nuclei hyperchromatic. The cytoplasmic changes were similar to those found in severe dysplasia. Nucleoli were not prominent. This cytological picture corresponded with sections showing very marked proliferation of basal and parabasal cells with thickening of the epithelium, but with the basal membrane remaining intact. The papillomatous projections of cells were larger and more cellular than in severe dysplasia, there was marked cell crowding and the nucleated malignant cells were present at the surface with no layer of keratin. The nuclei of the cells were enlarged and hyperchromatic and there were greatly increased numbers of mitotic figures at all levels.

At the stage of early invasive carcinoma the smears were very similar to those found in animals with in-situ carcinoma, except that neoplastic cells were found in larger numbers and included more atypical squamous cells from

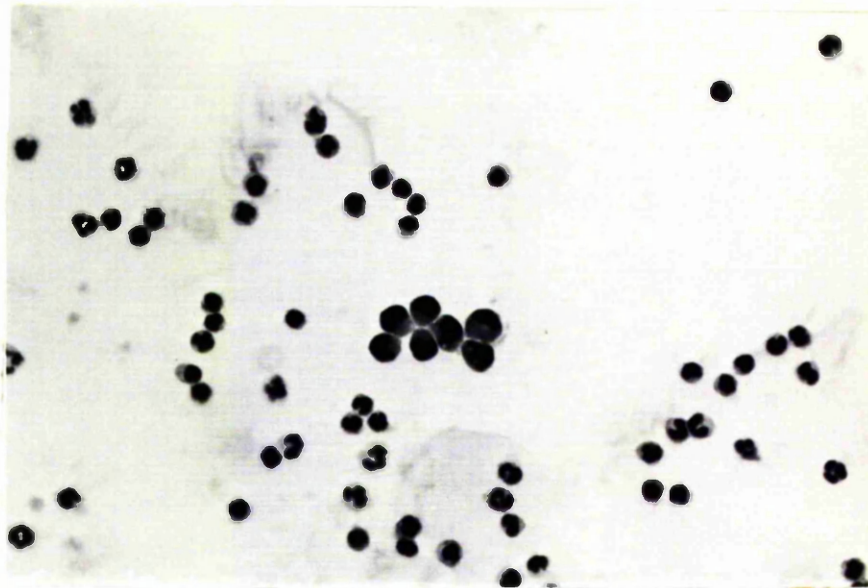


FIG. XV. Exfoliated basal and parabasal cells corresponding to early dysplasia.

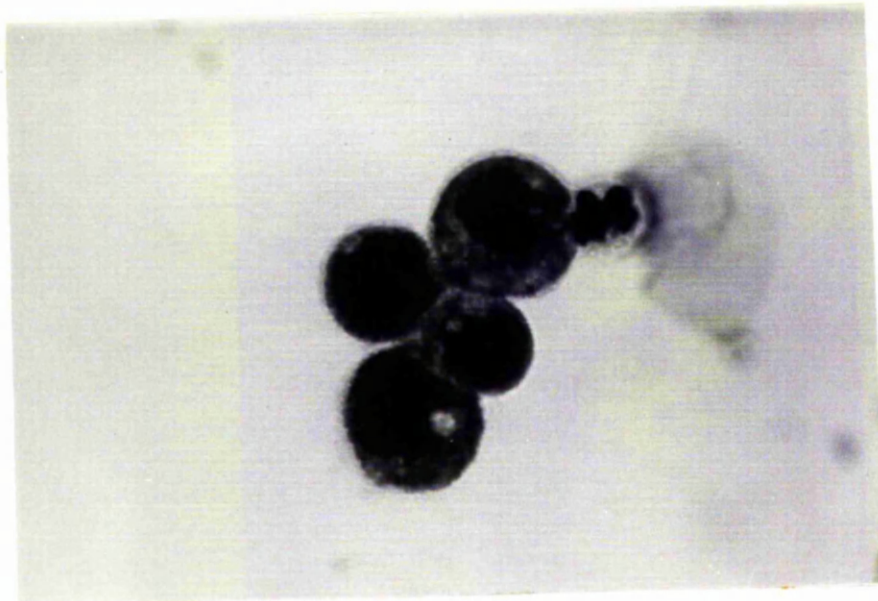


FIG. XVI. Plump hyperchromatic basal cells corresponding to more severe dysplasia.

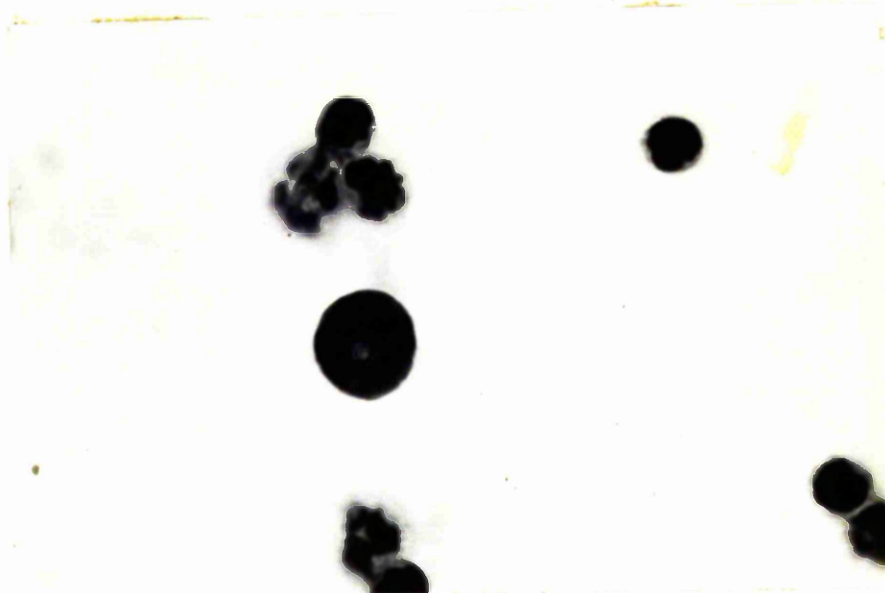


FIG. XVII. Cells with large nuclei, increased nucleo-cytoplasmic ratio and hyperchromatic nuclei : these cells were regarded as the first malignant cells.

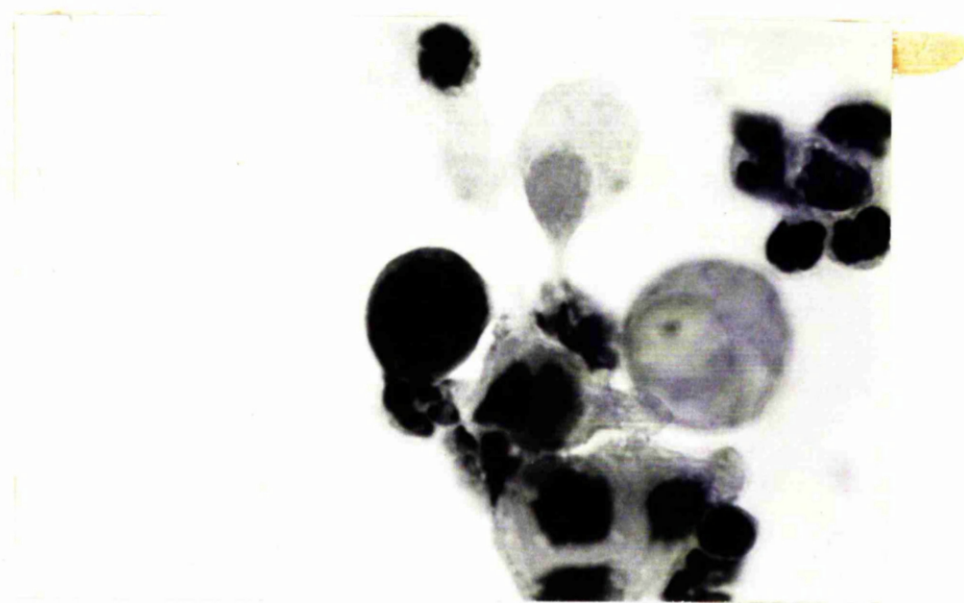


FIG. XVIII. Exfoliated cells corresponding with in-situ carcinoma.

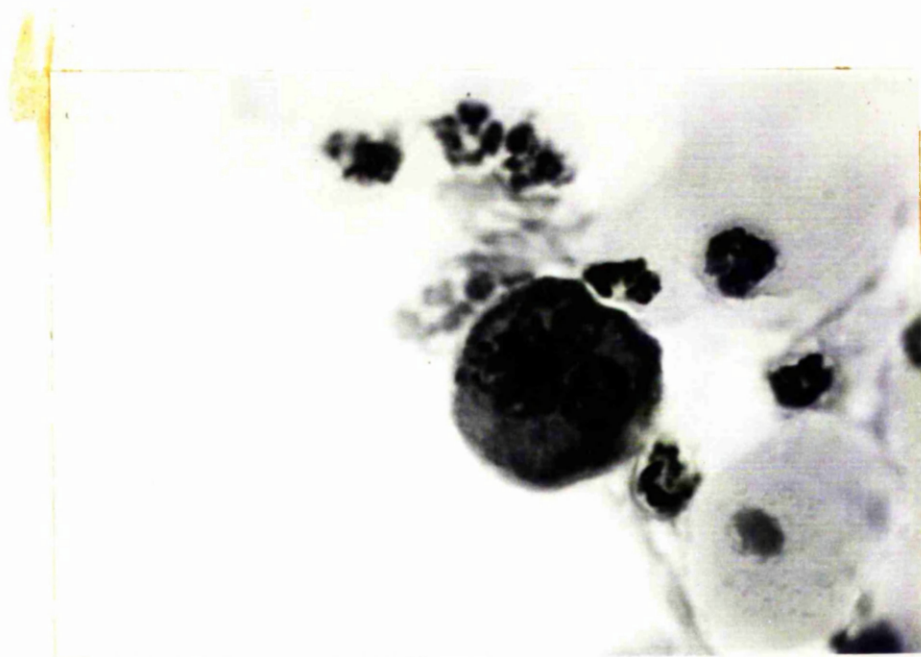


FIG. XIX. Multinucleated cell from in-situ carcinoma.

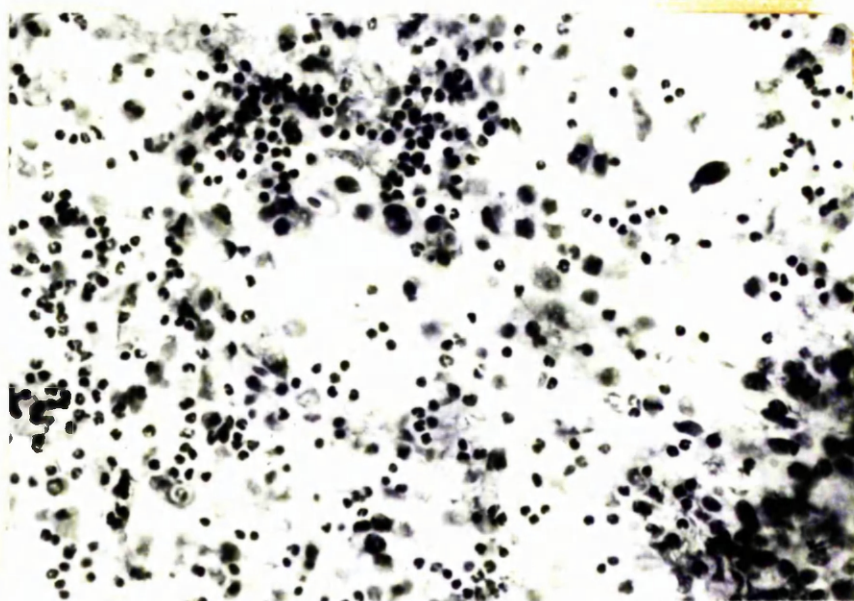


FIG. XX. Exfoliated cells corresponding to early invasive carcinoma with polymorphonuclear leukocytes and red blood cells.

the surface (Fig. XIX). Nucleoli were more prominent. At this stage too, polymorphonuclear leukocytes returned in large numbers and the smears contained red blood cells, but the essential difference between the invasive and the non-invasive lesion was quantitative. An important feature of the invasive lesion was that during the dysplastic and in-situ stage, the exfoliated cells remained cyclic, but when invasion occurred the cyclic pattern was distorted and basal and parabasal cells predominated with fewer keratinised and luteinised cells being exfoliated.

This correlation enabled us to be certain that the first cytologically malignant cells found were associated with definable histological lesions and that, with the relatively narrow range of variations in this model, it would be possible to assess what different histological stages had been reached, for the control of therapy.

One important point is that it was not possible to determine cytologically the exact time of breaching of the basement membrane, so that the term 'earliest invasion demonstrable' in this experiment means the earliest time we could be certain invasion was taking place rather than being synonymous with breaching of the basement membrane or 'micro-invasion'. Even within the relatively narrow range of variations in this experimental model we found it impossible to reliably detect micro-invasion cytologically, although this would have been the ideal.

A second point is that it was demonstrated that invasive carcinoma arose almost equally, in this model, from severe dysplasia and in-situ carcinoma.

(II) Preliminary experiments were also carried out to demonstrate that the tumours produced were immunogenic.

Tumours were induced in the cervix of two groups of four inbred Swiss virgin female mice, aged six to nine weeks, using the techniques previously described. One group was treated as in the ST experiment with the carcinogen remaining in position, the other as in the LT experiment in which the carcinogen was removed. Tissue from each tumour was used to immunise four separate groups of 12 syngeneic virgin female mice by subcutaneous (s.c.) inoculation of the tumour fragments by a trocar. A similar number of uninoculated animals served as controls. In the experimental group the growing tumour was excised after eight to ten days, and at the time of excision sham operations were performed in the controls.

Ten days after the removal of the tumour and the sham operation both groups of animals were irradiated and twenty-four hours later each group was challenged by the s.c. inoculation of 1×10^4 tumour cells suspended in 0.1 ml Eagles tissue culture medium, prepared by enzymatic (trypsin) digestion of tumour of the second transplant generation.

The animals were examined weekly for evidence of tumour outgrowth (the latent period) and at twenty weeks the diameter of the tumour measured.

In the group treated as the ST group, with the carcinogen remaining in position, of the four tumours tested three showed evidence of immunogenicity and in each of the cases all the animals showed resistance to tumour growth, in terms of latency and tumour growth compared to the controls.

The fourth tumour showed strong immunogenicity and all twelve experimental animals showed complete resistance to growth, while 11 of the 12 control animals developed tumours.

In the group treated as in the LT experiment with the carcinogen removed on the detection of the first malignant cells, with all four of the tumours tested there was more resistance to tumour growth in the experimental group than in the controls, but their differences were much less marked than in the ST group. In both experimental and control LT groups the latent period was longer than in the ST groups, and the ultimate size of the tumours smaller, suggesting that in comparison to the ST group the tumours were less immunogenic and slower growing.

A) The S.T. Group of experiments.

The results of the S.T. Group I experiments in which the carcinogen remained in position throughout and in which A.L.S. and N.H.S. were started, and the injection of B.C.G. or saline given, on day -14 relative to the time of carcinogen implantation, are given in Table I and Figure XXI.

These show that at the time of termination of the experiment there was a marked difference between the number of tumours developing in the mice receiving A.L.S., 15 out of 20 mice, (75%), and the mice receiving B.C.G., 8 out of 20 mice, (40%). However, when the number of mice with micro invasive carcinoma is included it is seen that the difference in the number of mice developing invasive carcinomas is much less marked; in the group receiving A.L.S. 16 out of 20 mice (80%) and in the B.C.G. group 13 out of 20 mice (65%), have developed invasive lesions. Severe dysplasia and/or carcinoma-in-situ, both epithelial forms from which invasive lesions can arise, are found in 4 out of 20 mice (20%) in the A.L.S. group and 5 out of 20 mice (25%) in the B.C.G. group, so that at the termination of this experiment, in the A.L.S. group, 20 out of 20 mice (100%) had either developed invasive malignancy or had epithelium which retained the potential to develop invasive carcinoma, while in the B.C.G. group 18 out of 20 mice (90%) had similar changes. In the control groups receiving N.H.S. or saline the figures were similar, suggesting that while the carcinogen remains in position, in experiments of short duration such as this, the effects of A.L.S. and B.C.G. are merely to increase or delay the rate of development of invasive carcinoma. This is emphasised by the finding that at the termination of the experiment there was no significant difference in the number of mice with normal epithelium, in either the experimental or control groups.

TABLE I : S.T. GROUP I.

Tumours ultimately developed in :

In the control group receiving N.H.S.,	12 out of 20 mice : 60%
In the experimental group receiving A.L.S.,	15 out of 20 mice : 75%
In the experimental group receiving B.C.G.,	8 out of 20 mice : 40%
In the group receiving saline,	11 out of 20 mice : 55%

Micro-invasive carcinoma was detected at post-mortem in :

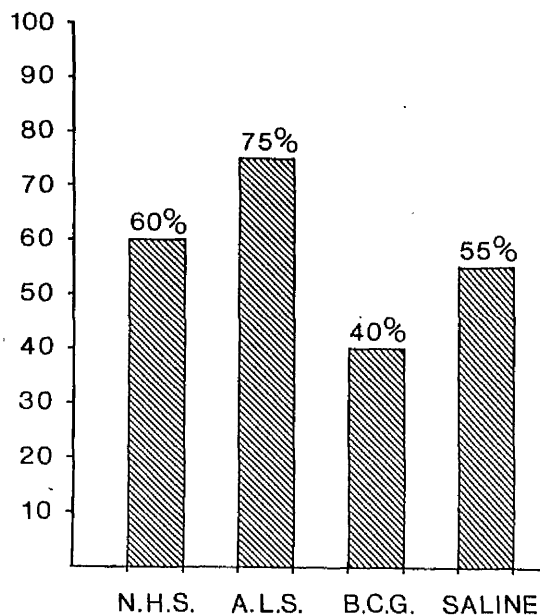
In the control group receiving N.H.S.,	3 out of 20 mice : 15%
In the experimental group receiving A.L.S.,	1 mouse out of 20 : 5%
In the experimental group receiving B.C.G.,	5 mice out of 20 : 25%
In the group of controls receiving saline,	3 mice out of 20 : 15%

Severe dysplasia and/or carcinoma in-situ was found at the post-mortem in :

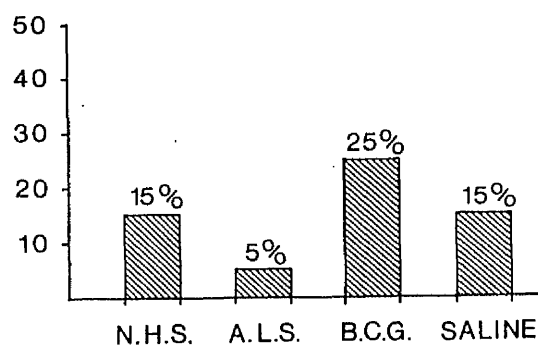
In the control group receiving N.H.S.,	4 out of 20 mice : 20%
In the experimental group receiving A.L.S.,	4 out of 20 mice : 20%
In the experimental group receiving B.C.G.,	5 out of 20 mice : 25%
In the control group receiving saline,	5 out of 20 mice : 25%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

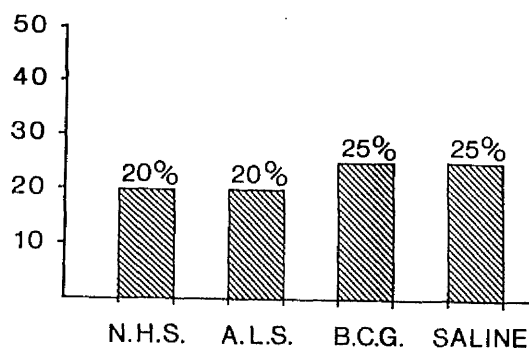
In the control group receiving N.H.S.,	1 out of 20 mice : 5%
In the experimental group receiving A.L.S.,	0 out of 20 mice : 0%
In the experimental group receiving B.C.G.,	2 out of 20 mice : 10%
In the control group receiving saline,	1 out of 20 mice : 5%



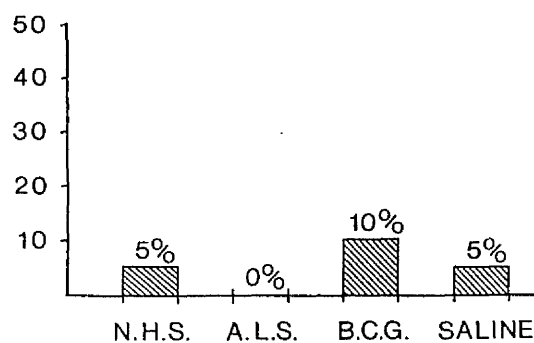
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXI. S.T.Group I : Carcinogen remaining in position throughout experiment.

A.L.S. and N.H.S. : Started day -14 relative to carcinogen implantation.

B.C.G. and saline : Given day -14 relative to carcinogen implantation.

In S.T. Group II where the A.L.S. and N.H.S. were started on the day of carcinogen implantation and in which B.C.G. or saline were given at this time the results are given in Table II and Figure XXII.

The results are very similar to those in S.T. Group I the only difference being that relatively more of the B.C.G. sub-group had either severe dysplasia or in-situ carcinoma at the termination of the experiment, 44%, suggesting more marked delay in the progression to invasive carcinoma, but the numbers with normal epithelium at the termination of the experiment, in both experimental and control groups, were the same.

When A.L.S. or B.C.G. was delayed until the first malignant cells were detected cytologically, S.T. Group III i.e. when the epithelium showed either severe dysplasia or in-situ carcinoma, the results in the different sub-groups and controls were similar to each other, (Table III and Figure XXIII) and were also similar to the other experimental groups S.T. I and S.T. II.

In S.T. Group IV where the A.L.S. and N.H.S. were started at the first cytological evidence of invasion and in which B.C.G. was started at the same time, the most significant finding was that 95% of the mice receiving A.L.S. had either tumours or early invasive lesions, compared with 78% of the mice receiving N.H.S., 49% of the mice receiving B.C.G. and 78% of the mice receiving saline. The other significant finding was that B.C.G. seemed capable of causing reversion of the lesion to a pre-invasive state, (39% of the mice), but not to a normal epithelium, in higher numbers than in the control groups. Table IV and Figure XXIV.

TABLE II : S.T. GROUP II

Tumours ultimately developed in :

In the control group receiving N.H.S.,	13 out of 24 mice : 54.16%
In the experimental group receiving A.L.S.,	12 out of 19 mice : 63.15%
In the experimental group receiving B.C.G.,	4 out of 18 mice : 22.22%
In the control group receiving saline,	8 out of 15 mice : 53.33%

Micro-invasive carcinoma was detected at post-mortem in:

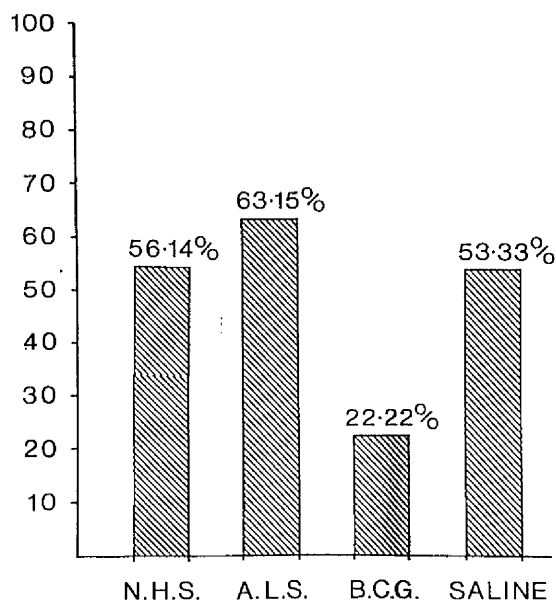
In the control group receiving N.H.S.,	4 out of 24 mice : 16.66%
In the experimental group receiving A.L.S.,	1 out of 19 mice : 5.26%
In the experimental group receiving B.C.G.,	4 out of 18 mice : 22.22%
In the control group receiving saline,	3 out of 15 mice : 20%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

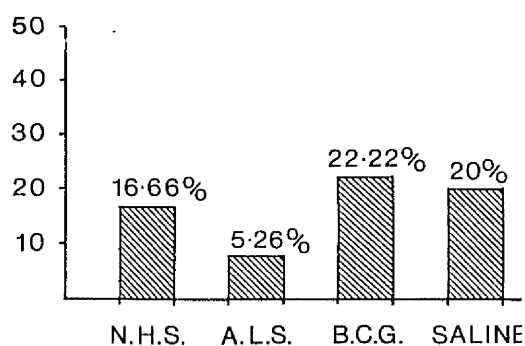
In the control group receiving N.H.S.,	7 out of 24 mice : 29.16%
In the experimental group receiving A.L.S.,	4 out of 19 mice : 21.05%
In the experimental group receiving B.C.G.,	8 out of 18 mice : 44.44%
In the control group receiving saline,	3 out of 15 mice : 20%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

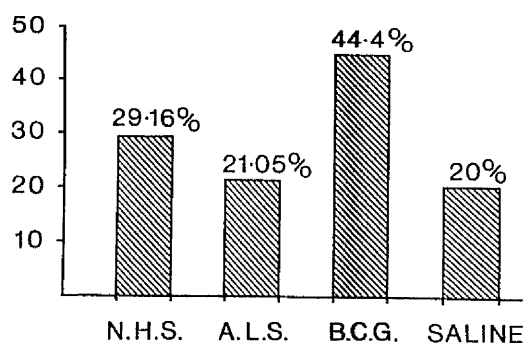
In the control group receiving N.H.S.,	0 out of 24 mice : 0%
In the experimental group receiving A.L.S.,	2 out of 19 mice : 10.52%
In the experimental group receiving B.C.G.,	2 out of 18 mice : 11.11%
In the control group receiving saline,	1 out of 15 mice : 6.66%



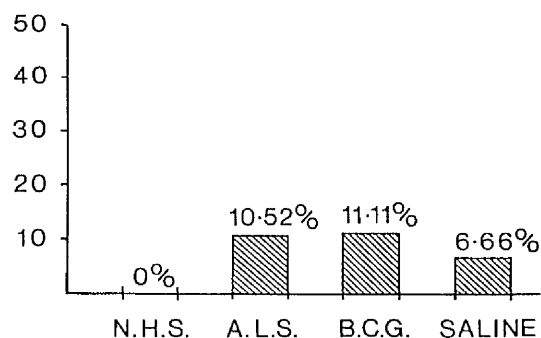
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXII. S.T. Group II : Carcinogen remaining in position throughout experiment.

A.L.S. and N.H.S. : Started on day of carcinogen implantation.

B.C.G. and saline : Given on day of carcinogen implantation.

TABLE III : S.T. GROUP III

Tumours ultimately developed in :

In the control group receiving N.H.S.,	8 out of 18 mice : 42.10%
In the experimental group receiving A.L.S.,	13 out of 19 mice : 68.42%
In the experimental group receiving B.C.G.,	5 out of 13 mice : 38.46%
In the control group receiving saline,	8 out of 15 mice : 53.33%

Micro-invasive carcinoma was detected at post-mortem in :

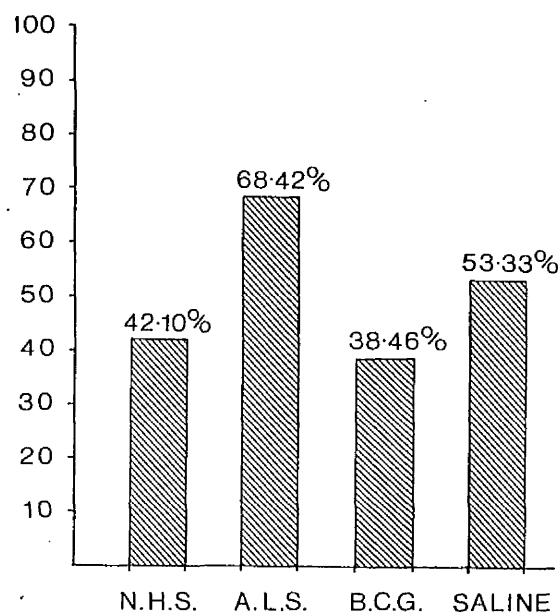
In the control group receiving N.H.S.,	4 out of 19 mice : 21.05%
In the experimental group receiving A.L.S.,	2 out of 19 mice : 10.52%
In the experimental group receiving B.C.G.,	3 out of 13 mice : 23.07%
In the control group receiving saline,	4 out of 15 mice : 26.66%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

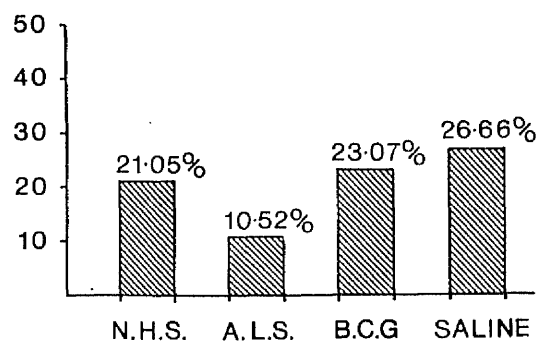
In the control group receiving N.H.S.,	5 out of 19 mice : 26.31%
In the experimental group receiving A.L.S.,	4 out of 19 mice : 21.05%
In the group receiving B.C.G.,	3 out of 13 mice : 23.07%
In the control group receiving saline,	2 out of 15 mice : 13.33%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

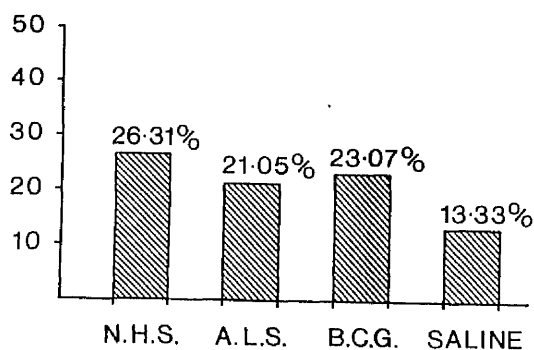
In the control group receiving N.H.S.,	2 out of 19 mice : 10.52%
In the experimental group receiving A.L.S.,	0 out of 19 mice : 0%
In the experimental group receiving B.C.G.,	2 out of 13 mice : 15.38%
In the control group receiving saline,	1 out of 15 mice : 6.66%



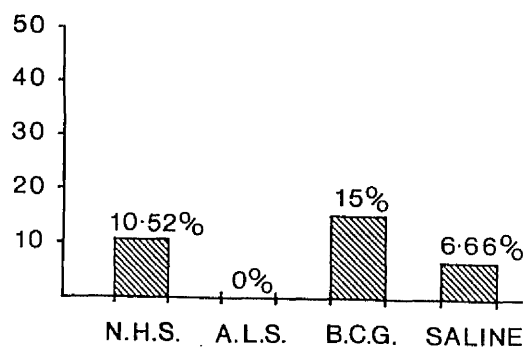
a) %Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or in-situ carcinoma.



c) %Mice with mild dysplasia or normal cervix.

FIG. XXIII. S.T. Group III : Carcinogen remaining in position throughout experiment.

A.L.S. and N.H.S. : Started at time of detection of first malignant cells by cytology.

B.C.G. and saline : Given on first detection of malignant cells by cytology.

TABLE IV. : S.T. GROUP IV.

Tumours ultimately developed in :

In the control group receiving N.H.S.,	8 out of 18 mice : 44.44%
In the experimental group receiving A.L.S.,	18 out of 22 mice : 81.81%
In the experimental group receiving B.C.G.,	6 out of 18 mice : 33.33%
In the control group receiving saline,	9 out of 16 mice : 56.25%

Micro-invasive carcinoma was detected at post-mortem in :

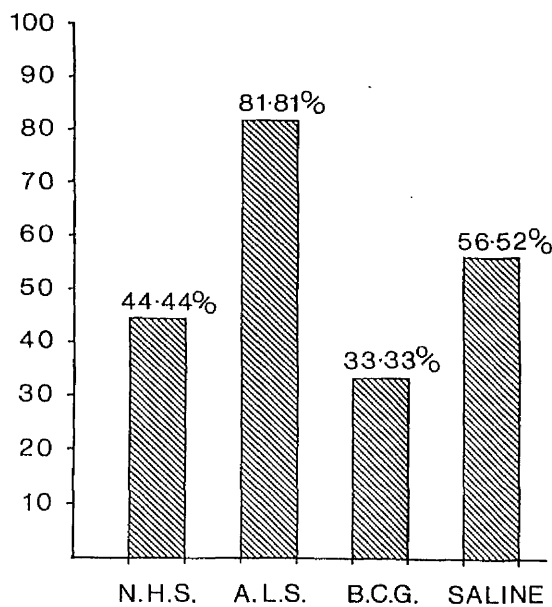
In the control group receiving N.H.S.,	6 out of 18 mice : 33.33%
In the experimental group receiving A.L.S.,	3 out of 22 mice : 13.63%
In the experimental group receiving B.C.G.,	3 out of 18 mice : 16.66%
In the control group receiving saline,	5 out of 16 mice : 31.25%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

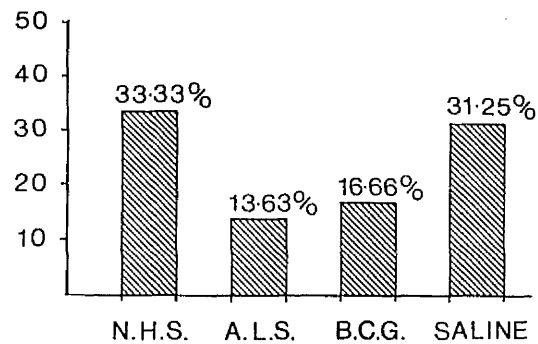
In the control group receiving N.H.S.,	3 out of 18 mice : 16.66%
In the experimental group receiving A.L.S.,	1 out of 22 mice : 4.54%
In the experimental group receiving B.C.G.,	7 out of 18 mice : 38.88%
In the control group receiving saline,	1 out of 16 mice : 6.25%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

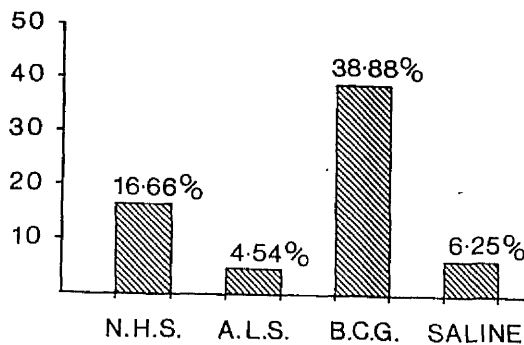
In the control group receiving N.H.S.,	1 out of 18 mice : 5.55%
In the experimental group receiving A.L.S.,	0 out of 22 mice : 0%
In the experimental group receiving B.C.G.,	2 out of 18 mice : 11.11%
In the control group receiving saline,	1 out of 16 mice : 6.25%



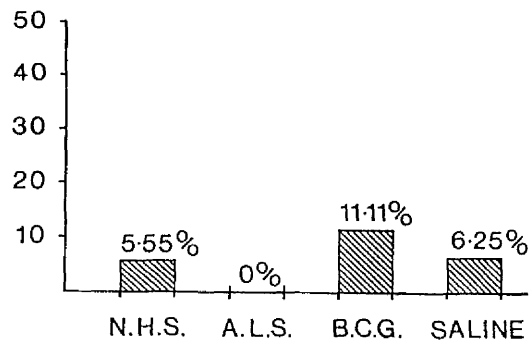
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or in-situ carcinoma.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXIV. S.T. Group IV : Carcinogen remaining in position throughout experiment.

A.L.S. and N.H.S. : Started at first evidence of invasion detected by cytology.

B.C.G. and saline : Given at first evidence of invasion detected by cytology.

B) The L.T. Group of experiment.

The results of the experiments in which the carcinogen was removed when the first malignant cells were detected cytologically, the L.T. experiments, are given in Tables V to VIII and Figures XXV to XXVIII.

In the L.T. group in which A.L.S. and N.H.S. were started on the day of implantation of the carcinogen and the injection of B.C.G. or saline given at that time there was a marked difference in the number of mice developing tumours between the group receiving A.L.S. 12 out of 18 mice, 66%, and the controls receiving N.H.S., 8 out of 16 mice, 50%. Of the experimental group receiving B.C.G. 6 out of 21 mice, 28.5%, developed tumours whereas in the control group receiving saline 8 out of 18 mice, 44.5%, developed tumours (Table V and Fig. XXV). From these it can also be seen that the percentage of mice, receiving A.L.S. developing tumours was also markedly increased over the group receiving B.C.G.

When the numbers of mice developing micro-invasive carcinoma is included it can be seen that in contrast to the S.T. experiments the difference in the numbers of mice developing invasive lesions between the group receiving A.L.S. 15 out of 18 mice, 92%, and the group receiving B.C.G. 7 out of 21 mice, 33% is appreciable. This contrasting effect of A.L.S. and B.C.G., when the carcinogen has been removed is emphasised when the number of mice with tumours, early invasive carcinoma, or severe dysplasia and carcinoma in-situ, is demonstrated at the termination of the experiment, when it can be seen that 17 out of 18 mice, 94.5%, receiving A.L.S. had either developed, or retained the potential to develop, invasive carcinoma whereas in the group receiving B.C.G. 10 out of 21 mice, 47.5%, and in the control groups 12 out of 16, 75%, and 14 out of 18 mice, 77.5%, were in this category.

TABLE V : L.T. GROUP I

Tumours ultimately developed in :

In the control group receiving N.H.S.,	8 out of 16 mice : 50%
In the experimental group receiving A.L.S.,	12 out of 18 mice : 66.66%
In the experimental group receiving B.C.G.,	6 out of 21 mice : 28.57%
In the control group receiving saline,	8 out of 18 mice : 44.44%

Micro-invasive carcinoma was detected at post-mortem in :

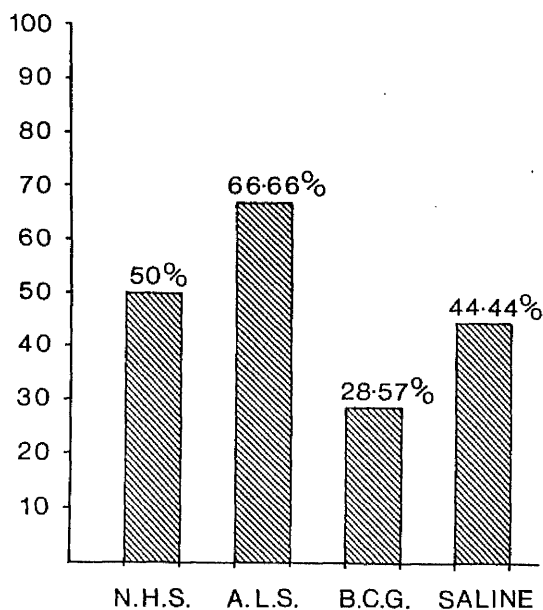
In the control group receiving N.H.S.,	2 out of 16 mice : 12.5%
In the experimental group receiving A.L.S.,	3 out of 18 mice : 16.66%
In the experimental group receiving B.C.G.,	1 out of 21 mice : 4.76%
In the control group receiving saline,	2 out of 18 mice : 11.11%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

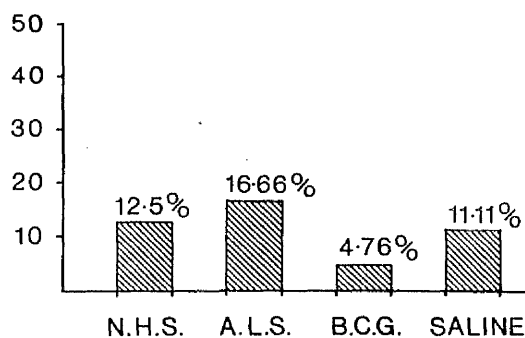
In the control group receiving N.H.S.,	2 out of 16 mice : 12.5%
In the experimental group receiving A.L.S.,	2 out of 18 mice : 11.11%
In the experimental group receiving B.C.G.,	3 out of 21 mice : 14.28%
In the control group receiving saline,	4 out of 18 mice : 22.22%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

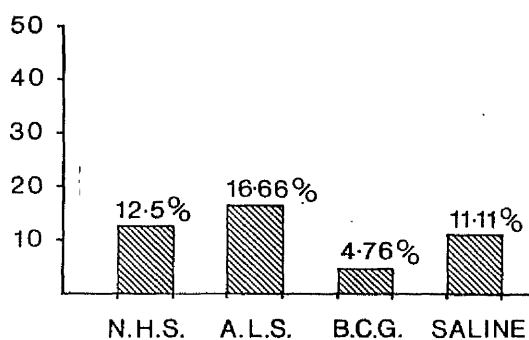
In the control group receiving N.H.S.,	4 out of 16 mice : 25%
In the experimental group receiving A.L.S.,	1 out of 18 mice : 5.55%
In the experimental group receiving B.C.G.,	11 out of 21 mice : 52.3%
In the control group receiving saline,	4 out of 18 mice : 22.22%



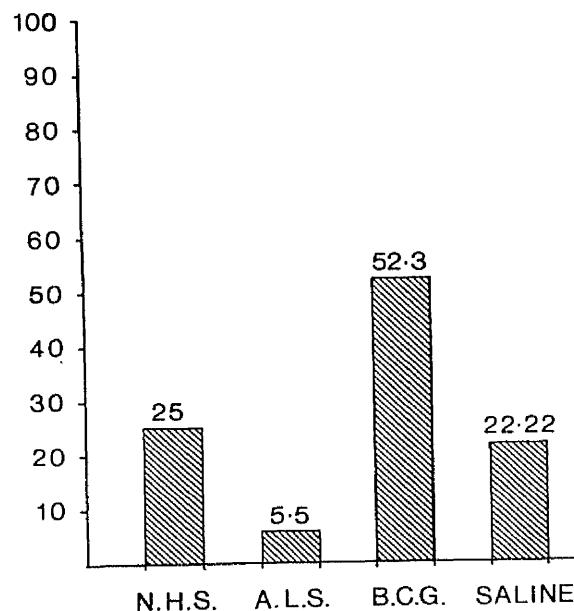
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXV. L.T. Group I : Carcinogen removed when first malignant cells detected by exfoliative cytology.

A.L.S. and N.H.S. : Started on day of implantation of carcinogen.

B.C.G. and saline : Given on day of implantation of carcinogen.

Further emphasis for the contrasting effects of A.L.S. and B.C.G. when the carcinogen has been removed is found in the figures for the number of cervixes which had returned to normal at the end of the experiment, in the A.L.S. group only 1 out of 18 mice, 5.5%, had an apparently normal cervix whereas in the B.C.G. group 11 out of 21 mice, 52%, had reverted to normal. In the control groups 4 out of 16 mice, 25%, and 4 out of 18 mice, 22%, had returned to normal from a potentially invasive epithelium.

In L.T. group 2, in which A.L.S. and N.H.S. were started on the day of removal of the carcinogen and B.C.G. and saline injections were given that day the figures for all the groups were similar to those of the L.T. group 1, and no effect could be demonstrated, of the delay in giving A.L.S. or B.C.G. (Table VI and Fig. XXVI).

These experiments emphasise that when the carcinogen is removed A.L.S. or B.C.G. have a marked effect on the progression or regression of potentially invasive epithelium. In this situation A.L.S. prevents the reversion of the epithelium to normal and promotes the development of invasive carcinoma. In contrast B.C.G. increases the number of cervixes reverting to normal in comparison to the controls and A.L.S. groups, and also decreases the number of carcinomas forming.

In L.T. groups 3 and 4 an attempt was made to further reduce the influence of the carcinogen by waiting three months after removal of the carcinogen and sub-dividing the mice into two experimental groups, L.T. group 3 with cytological evidence of in-situ malignancy, and L.T. group 4 with cytological evidence of early invasive carcinoma but with no visible or palpable tumour.

TABLE VI : L.T. GROUP 2

Tumours ultimately developed in :

In the control group receiving N.H.S.,	9 out of 21 mice : 42.85%
In the experimental group receiving A.L.S.,	12 out of 16 mice : 75%
In the experimental group receiving B.C.G.,	5 out of 18 mice : 27.77%
In the control group receiving saline,	10 out of 20 mice : 50%

Micro-invasive carcinoma was detected at post-mortem in :

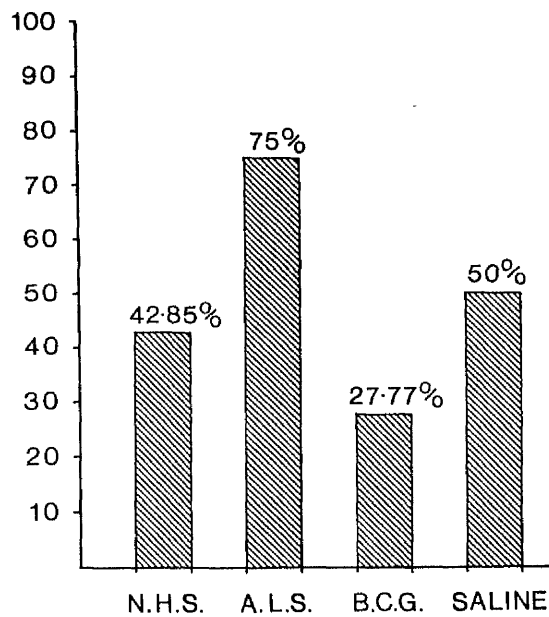
In the control group receiving N.H.S.,	4 out of 21 mice : 19.04%
In the experimental group receiving A.L.S.,	1 out of 16 mice : 6.25%
In the experimental group receiving B.C.G.,	3 out of 18 mice : 16.66%
In the control group receiving saline,	2 out of 20 mice : 10%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

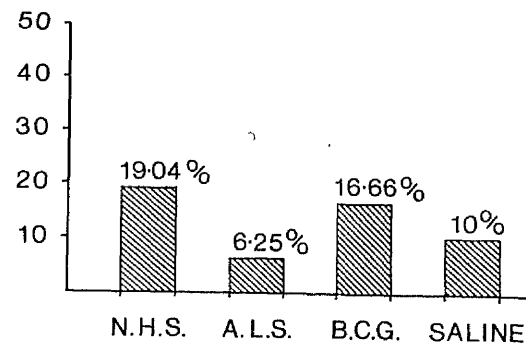
In the control group receiving N.H.S.,	4 out of 21 mice : 19.04%
In the experimental group receiving A.L.S.,	1 out of 16 mice : 6.25 %
In the experimental group receiving B.C.G.,	2 out of 18 mice : 11.11%
In the control group receiving saline,	5 out of 20 mice : 25%

A normal cervix or a cervix showing mild dysplasia was found at post mortem in :

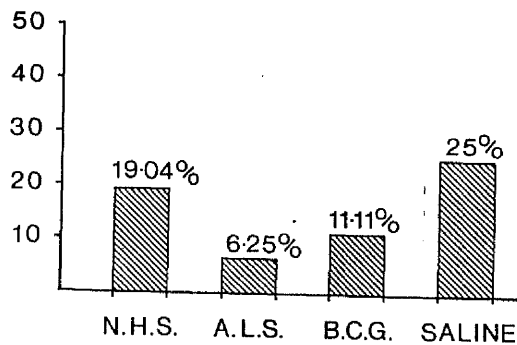
In the control group receiving N.H.S.,	4 out of 21 mice : 19.04%
In the experimental group receiving A.L.S.,	2 out of 16 mice : 12.5%
In the experimental group receiving B.C.G.,	8 out of 18 mice : 44.44%
In the control group receiving saline,	3 out of 20 mice : 15%



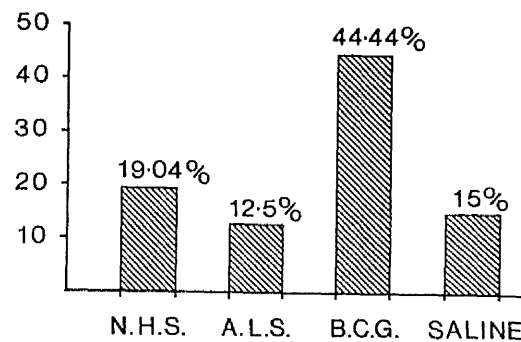
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXVI. L.T. Group 2 : Carcinogen removed when first malignant cells detected by exfoliative cytology.

A.L.S., and N.H.S. : Started on day of removal of carcinogen.

B.C.G. and saline : Given on day of removal of carcinogen.

In L.T. group 3 the results (Table VII and Fig. XXVII) were essentially the same as in L.T. groups 1 and 2, and demonstrated a marked effect of A.L.S. by the increased incidence of tumours and in the maintenance of an in-situ malignancy and a corresponding failure of the reversal of the epithelium to normal with none of the mice receiving A.L.S. having a normal epithelium at the termination of the experiment.

B.C.G. had a marked contrasting effect with only 3 out of 16 mice, 18.7 %, forming tumours and 10 out of 16 mice, 62.5%, reverting to a normal epithelium while in the control groups receiving N.H.S., 3 out of 20 mice, 15%, or saline, 4 out of 19 mice, 21%, reverted to normal.

In L.T. group 4 the effect of immune stimulation and immuno-suppression on early invasive carcinoma was demonstrated (Table VIII and Fig. XXVIII). As might have been expected the incidence of tumours developing in the controls was higher than in the other L.T. groups being 60% and 69.5% in the N.H.S. and saline control groups respectively.

In the experimental group receiving A.L.S. 13 out of 15 mice, 86.6%, ultimately developed tumours and 1 out of 15 mice, 6.6%, had a micro-invasive carcinoma. One mouse had an in-situ preinvasive lesion and none of the mice had a normal cervix at the termination of the experiment.

Of the group receiving B.C.G. 2 out of 15 mice developed tumours and 3 out of 15 mice had micro-invasive carcinoma at the termination of the experiment giving an incidence of 5 out of 15 mice, 33.3%, with definite invasive lesions. Three out of 15 mice, 20%, had either severe dysplasia or in-situ carcinoma and 7 out of 15 mice, 46.5%, had mild dysplasia or normal epithelium, suggesting that with early invasive lesions B.C.G. could be effective in preventing tumour development and could cause reversal of the malignant epithelium to normal. In this group none of the control mice reverted to normal.

TABLE VII : L.T. GROUP 3

Tumours ultimately developed in :

In the control group receiving N.H.S.,	9 out of 20 mice : 45%
In the experimental group receiving A.L.S.,	13 out of 18 mice : 72.22%
In the experimental group receiving B.C.G.,	3 out of 16 mice : 18.75%
In the control group receiving saline,	8 out of 19 mice : 42.10%

Micro-invasive carcinoma was detected at post-mortem in :

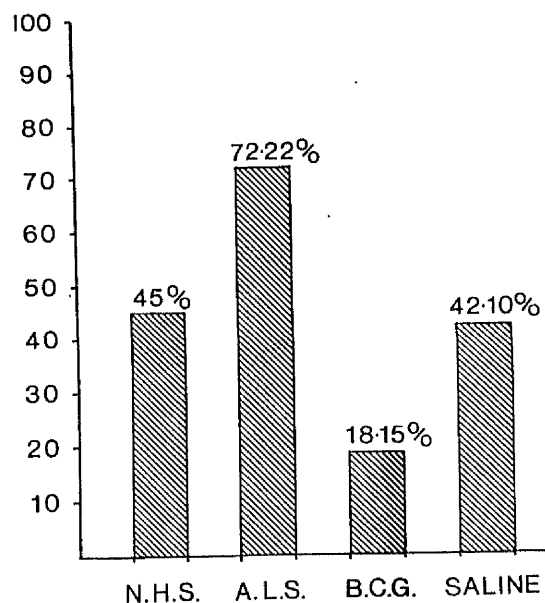
In the control group receiving N.H.S.,	3 out of 20 mice : 15%
In the experimental group receiving A.L.S.,	2 out of 18 mice : 11.11%
In the experimental group receiving B.C.G.,	1 out of 16 mice : 6.25%
In the control group receiving saline,	3 out of 19 mice : 15.78%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

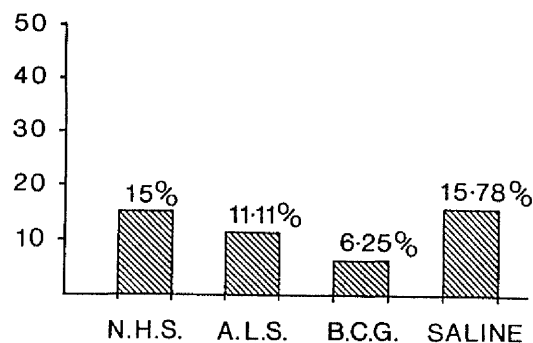
In the control group receiving N.H.S.,	5 out of 20 mice : 25%
In the experimental group receiving A.L.S.,	3 out of 18 mice : 16.66%
In the experimental group receiving B.C.G.,	2 out of 16 mice : 12.5%
In the control group receiving saline,	4 out of 19 mice : 21.05%

A normal cervix showing mild dysplasia was found in:

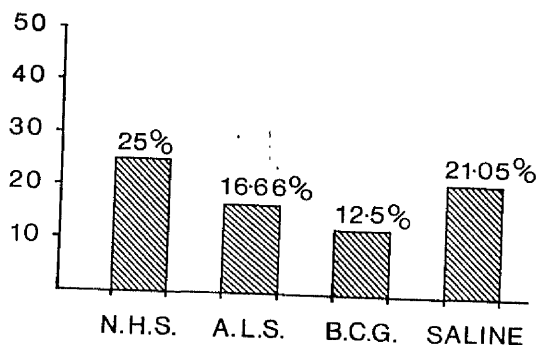
In the control group receiving N.H.S.,	3 out of 20 mice : 15%
In the experimental group receiving A.L.S.,	0 out of 18 mice : 0%
In the experimental group receiving B.C.G.,	10 out of 16 mice : 62.5%
In the control group receiving saline,	4 out of 19 mice : 21.05%



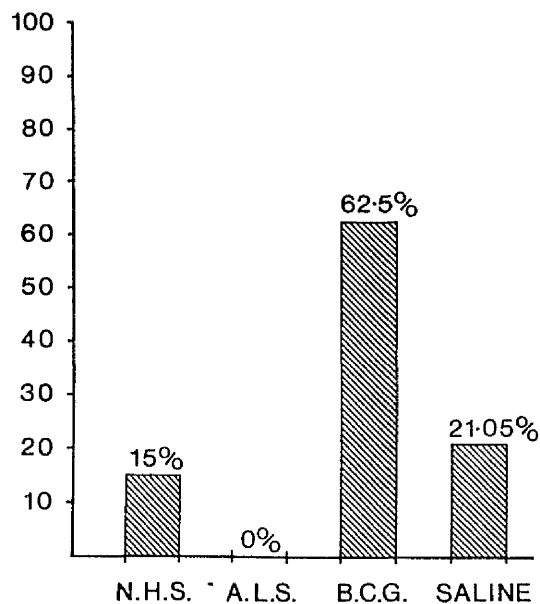
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXVII. L.T. Group 3 : Carcinogen removed when first malignant cells detected. 3 months after removal of carcinogen mice with evidence of in-situ carcinoma and/or severe dysplasia, but with no evidence of invasion grouped into this experiment.

A.L.S. and N.H.S. : Started 3 months after removal of carcinogen.

B.C.G. and saline : Given 3 months after removal of carcinogen.

TABLE VIII : L.T. GROUP 4

Tumours ultimately developed in :

In the control group receiving N.H.S.,	9 out of 15 mice : 60%
In the experimental group receiving A.L.S.,	13 out of 15 mice : 86.66%
In the experimental group receiving B.C.G.,	2 out of 15 mice : 13.33%
In the control group receiving saline,	10 out of 16 mice : 62.5%

Micro-invasive carcinoma was detected at post-mortem in :

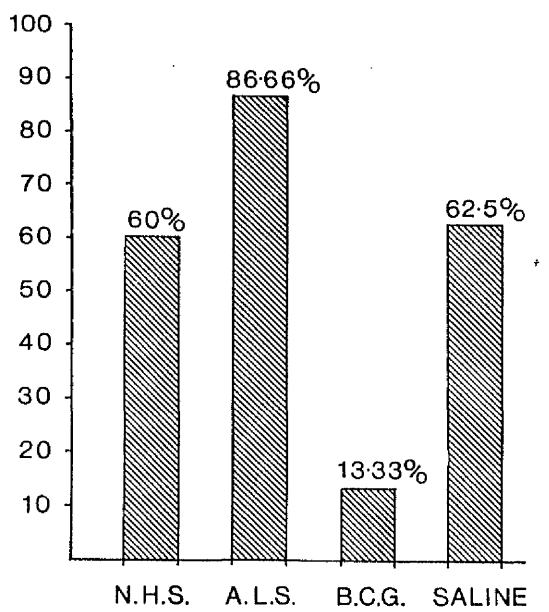
In the control group receiving N.H.S.,	4 out of 15 mice : 26.66%
In the experimental group receiving A.L.S.,	1 out of 15 mice : 6.66%
In the experimental group receiving B.C.G.,	3 out of 15 mice : 20%
In the control group receiving saline,	5 out of 16 mice : 31.25%

Severe dysplasia and/or carcinoma in-situ was found at post-mortem in :

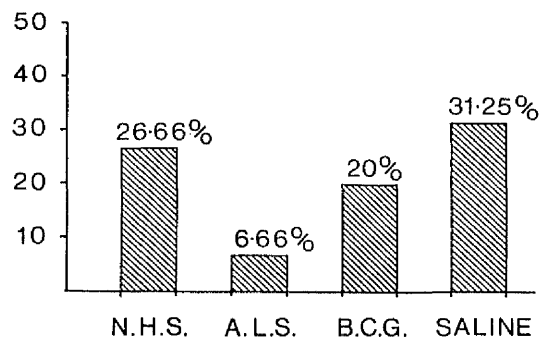
In the control group receiving N.H.S.,	2 out of 15 mice : 13.33%
In the experimental group receiving A.L.S.,	1 out of 15 mice : 6.66%
In the experimental group receiving B.C.G.,	3 out of 15 mice : 20%
In the control group receiving saline,	1 out of 16 mice : 6.25%

A normal cervix or a cervix showing mild dysplasia was found at post-mortem in :

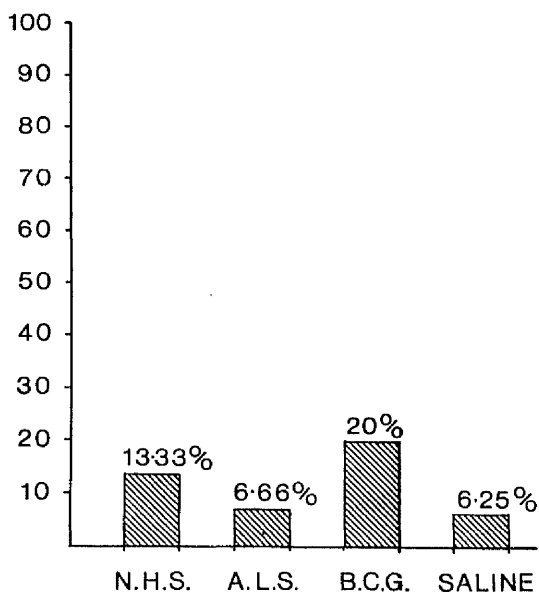
In the control group receiving N.H.S.,	0 out of 15 mice : 0%
In the experimental group receiving A.L.S.,	0 out of 15 mice : 0%
In the experimental group receiving B.C.G.,	7 out of 15 mice : 46.66%
In the control group receiving saline,	0 out of 16 mice : 0%



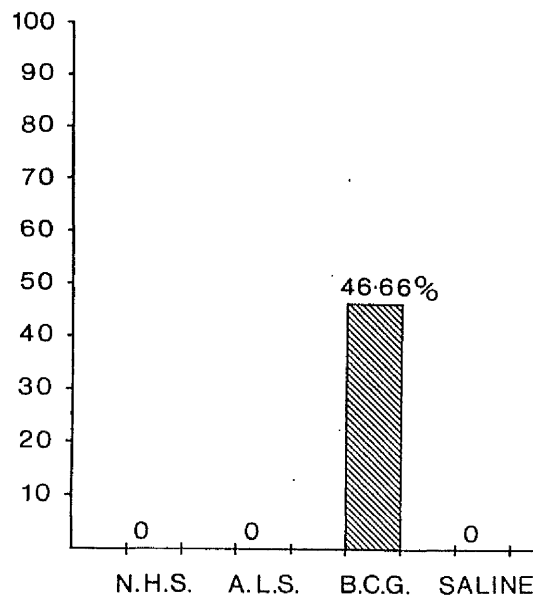
a) % Mice developing tumours.



b) % Mice with micro-invasive carcinoma.



c) % Mice with severe dysplasia and/or carcinoma in-situ.



d) % Mice with mild dysplasia or normal cervix.

FIG. XXVIII. L.T. Group 4 : Carcinogen removed when first malignant cells detected. 3 months after removal of carcinogen mice with cytological evidence of invasion, but no palpable tumour, grouped into this experiment.

A.L.S. and N.H.S. : Started 3 months after removal of carcinogen.

B.C.G. and saline : Given 3 months after removal of carcinogen.

C) The length of time from carcinogen implantation to the detection of the first malignant cells.

Details of the results of the experiment designed to determine the effect of A.L.S. and B.C.G. on the time from the implantation of the carcinogen to the detection of the first cytologically malignant cells are shown in Table IX. The animals were from the S.T. I, S.T. II, and L.T. I and L.T. 2 experiments.

Of the groups receiving N.H.S., 55 animals produced malignant cells and were available for statistical analysis. This showed a mean time to the production of malignant cells of 7.18 weeks with a standard deviation (S.D.) of 2.0.

In the A.L.S. group the 54 animals included for analysis had a mean time to the production of malignant cells of 7.18 weeks (S.D. 2.0).

In the group receiving B.C.G. the 44 animals included had a mean time to the first detection of malignant cells of 6.8 weeks (S.D. 2.22).

In the saline group the 47 animals had a mean time of 7.19 weeks (S.D. 2.36) to the production of malignant cells.

From these figures it can be seen that when the results for the animals producing malignant cells are examined there is no significant difference between the control groups and the experimental groups, nor between the two experimental groups, when the carcinogen remains in position.

TABLE IX. TIME FROM CARCINOGEN IMPLANTATION TO DETECTION OF MALIGNANT CELLS.

Treatment	No. of animals	No. of weeks	Mean \bar{x}	S.D. $\sqrt{\sigma_n^2}$
N.H.S.	55	7; 9; 7; 4; 8; 11; 6; 5; 6; 8; 7; 9; 5; 8; 4; 9; 11; 7; 5; 8; 8; 5; 7; 9; 8; 4; 5; 7; 6; 5; 6; 4; 11; 7; 9; 8; 7; 8; 8; 9; 5; 5; 5; 7; 9; 10; 11; 10; 6; 8; 6; 11; 6; 5;	7.18	2.0
A.L.S.	54	8; 8; 4; 9; 5; 2; 5; 5; 7; 9; 11; 7; 6; 6; 12; 7; 8; 7; 7; 9; 6; 6; 7; 11; 10; 5; 5; 4; 8; 8; 9; 5; 7; 8; 6; 5; 6; 9; 11; 9; 10; 7; 5; 8; 5; 7; 10; 9; 6; 5; 8; 7; 8;	7.18	2.0
B.C.G.	44	5; 9; 10; 8; 8; 4; 7; 3; 9; 7; 6; 6; 8; 10; 7; 5; 7; 12; 3; 6; 6; 6; 3; 7; 12; 10; 5; 7; 6; 8; 6; 7; 3; 7; 9; 4; 9; 8; 6; 8; 5; 5; 5; 8;	6.8	2.22
SALINE	47	6; 9; 9; 3; 5; 7; 7; 4; 11; 12; 8; 5; 2; 6; 6; 7; 9; 8; 8; 9; 4; 12; 7; 5; 3; 7; 9; 6; 9; 8; 11; 8; 7; 4; 7; 6; 5; 6; 8; 8; 8; 9; 7; 8; 10; 11; 4;	7.19	2.36

D) The length of time from the implantation of the carcinogen to the earliest evidence of invasion.

The experiment designed to determine the time from the carcinogen implantation to the first evidence of invasion, and the effect thereon of B.C.G. immune stimulation and A.L.S. immune suppression, was divided into two parts : the first part included mice from the groups S.T. I and S.T. II and in this section the carcinogen remained in position throughout. Details of the results are shown in Table X.

In this experiment, of those animals receiving N.H.S., 32 produced evidence of invasion and were included in the statistical analysis which gave a mean time to invasion of 13.9 weeks (S.D. 2.23).

Of the animals receiving A.L.S. 29 produced evidence of invasion with a mean time to invasion of 13.4 weeks (S.D. 1.93).

In the group receiving B.C.G. the mean time to invasion, of the 21 animals producing evidence of invasion, was 16.47 weeks (S.D.2.4).

The 25 animals included in the saline control group had a mean time to invasion of 14.28 weeks (S.D. 1.79).

From these figures it can be seen that, when the carcinogen remains in position and tumours are produced over a relatively short period there is no evidence of any significant acceleration or delay of invasion by A.L.S. or B.C.G. when compared to each other, or to the control groups.

TABLE X. TIME FROM CARCINOGEN IMPLANTATION TO DETECTION OF INVASION (CARCINOGEN REMAINING IN POSITION).

Treatment	No. of animals	No. of weeks	Mean \bar{x}	S.D. σ_{n-1}
N.H.S.	32	13; 14; 12; 18; 13; 13; 15; 9; 14; 12; 15; 17; 14; 13; 15; 15; 14; 18; 16; 14; 14; 15; 13; 17; 15; 12; 9; 9; 15; 13;	13.9	2.23
A.L.S.	29	12; 13; 10; 13; 10; 16; 15; 14; 14; 15; 14; 16; 12; 18; 13; 11; 12; 14; 15; 13; 12; 14; 15; 12; 11; 16; 14; 14; 11;	13.4	1.93
B.C.G.	21	15; 17; 15; 21; 17; 18; 16; 17; 14; 14; 17; 18; 19; 19; 16; 13; 12; 14; 21; 16; 17;	16.47	2.4
SALINE	25	15; 14; 17; 13; 12; 14; 14; 15; 14; 15; 16; 13; 12; 16; 16; 15; 12; 13; 13; 12; 12; 14; 15; 19; 16;	14.28	1.79

The second part of this experiment included animals from the groups L.T. 1 and L.T. 2 in which the carcinogen was removed on the first detection of malignant cells by cytological examination. The results are shown in detail in Table XI.

In this experiment, of the animals receiving N.H.S. 23 were suitable for statistical analysis having produced evidence of invasion. In these animals the mean time to invasion was 34.0 weeks (S.D. 4.0).

Of the animals receiving A.L.S., 28 produced evidence of invasion and statistical analysis showed a mean time to invasion of 26 weeks (S.D. 5.2).

The B.C.G. group contained 15 animals that produced evidence of invasion with a mean time to invasion of 40.9 weeks (S.D. 6.0).

In the saline control group the 22 animals included with evidence of invasion had a mean time to invasion of 34.8 weeks (S.D. 3.78).

These results show that when the influence of the carcinogen is removed there is a significant shortening of the length of time to the occurrence of invasion when the animals receiving A.L.S. are compared to the control groups ($P < 0.02$), and that there is a significant lengthening of the length of time to the occurrence of invasion when the group stimulated by B.C.G. is compared to the control groups ($P < 0.02$). In this experiment it is also demonstrated that the difference between the A.L.S. immunosuppressed group and the B.C.G. immunostimulated group is highly significant ($P < 0.001$).

TABLE XI. TIME FROM CARCINOGEN IMPLANTATION TO INVASION (CARCINOGEN REMOVED ON DETECTION OF FIRST MALIGNANT CELLS).

Treatment	No. of animals	No. of weeks	Mean \bar{x}	S.D. σ_{n-1}
N.H.S.	23	29; 31; 35; 31; 41; 36; 38; 41; 32; 33; 33; 37; 29; 37; 39; 38; 28; 32; 30; 31; 36; 37; 29;	34.0	4.0
A.L.S.	28	26; 27; 21; 27; 35; 19; 24; 24; 31; 36; 21; 28; 35; 27; 31; 17; 22; 21; 23; 26; 25; 24; 19; 31; 32; 22; 26; 21;	26.0	5.2
SALINE	22	39; 33; 37; 35; 41; 33; 33; 27; 33; 34; 35; 32; 37; 42; 36; 32; 41; 37; 31; 34; 29; 35;	34.8	3.78
B.C.G.	15	41; 35; 39; 43; 47; 29; 37; 43; 49; 38; 45; 31; 44; 45; 48;	40.9	6.0

E) The time from detection of the first malignant cells to the detection of invasion.

This experiment to determine the length of time from the detection of the first malignant cells to the first evidence of invasion, and the effect on this of A.L.S. or B.C.G. treatment was divided into two parts.

In the first part, using the animals from the S.T. I and S.T. II groups, in which the carcinogen remained in position throughout, the following results were obtained (Table XII).

Of the animals receiving N.H.S., 32 were available and these showed a mean time of 7.15 weeks (S.D. 2.87) from detection of first malignant cells till the occurrence of invasion.

29 animals receiving A.L.S. had evidence of invasion and the mean time from the detection of first malignant cells to invasion was 6.6 weeks (S.D. 2.4).

In the group receiving B.C.G. the 21 animals had a mean time of 9 weeks from first malignant cells to the earliest detection of invasion.

Of the saline control group the 25 animals which produced evidence of invasion had a mean time of 7.4 weeks (S.D. 3.18) from first malignant cells to the earliest detectable signs of invasion.

These results show a slight lengthening of the time from the first detection of malignant cells to the earliest signs of invasion in the group receiving B.C.G., compared to both the controls and the A.L.S. group but this difference was not significant ($P < 0.2$).

TABLE XII. TIME FROM DETECTION OF FIRST MALIGNANT CELLS TO DETECTION OF INVASION (CARCINOGEN IN POSITION THROUGHOUT).

Treatment	No. of animals	No. of weeks	Mean \bar{x}	S.D. σ_{n-1}
N.H.S.	32	6; 5; 5; 14; 5; 2; 9; 4; 8; 8; 9; 5; 11; 6; 3; 11; 6; 6; 9; 8; 6; 12; 8; 6; 3; 4; 9; 9;	7.15	2.87
A.L.S.	29	4; 5; 6; 4; 5; 14; 10; 9; 7; 5; 4; 7; 10; 6; 6; 6; 3; 5; 8; 4; 6; 8; 9; 5; 5; 6; 9; 9; 7;	6.6	2.4
B.C.G.	21	10; 8; 5; 13; 9; 14; 9; 4; 5; 7; 11; 12; 11; 9; 9; 8; 5; 2; 18; 10; 11;	9	3.69
SALINE	25	9; 5; 8; 10; 7; 7; 11; 3; 3; 8; 8; 10; 10; 8; 3; 5; 5; 3; 8; 2; 8; 14; 13;	7.4	3.18

In the second part of this experiment animals from groups L.T. 1 and L.T. 2, in which the carcinogen was removed on the detection of the first malignant cells, were used.

The details of the results are given in Table XIII.

Of the animals receiving N.H.S., 23 were available, having produced evidence of invasion, and in this group the mean time from the detection of the first malignant cells to invasion was 27 weeks (S.D. 4.59).

Of the animals receiving A.L.S., 28 produced evidence of invasion, with a mean time of 18.6 weeks (S.D. 5.18) from first malignant cells to the occurrence of invasion.

In the group receiving B.C.G. statistical analysis of the 15 animals that produced evidence of invasion showed a mean time of 34.3 weeks (S.D. 6.0) from the detection of the first malignant cells to the demonstration of invasion.

The 22 animals in the saline control group had a mean time of 27.4 weeks (S.D. 5.0) from first detected malignant cells to invasion.

These results further emphasise the effect of B.C.G. and A.L.S. on the progression of carcinogenesis from the cytologically malignant in-situ stage to the early invasive carcinoma, where the influence of the carcinogen has been removed. The reduction in the mean time from the detection of the first malignant cells to invasion in the group receiving A.L.S. is significant compared to the control groups ($P < 0.01$). Contrasting with this, the lengthening of the time from the detection of first malignant cells to the occurrence of invasion in the B.C.G. group compared to the controls is also significant.

The difference in the time from the detection of the first malignant cells to invasion when the A.L.S. group and the B.C.G. group are compared is highly significant ($P < 0.001$).

TABLE XIII. TIME FROM DETECTION OF FIRST MALIGNANT CELLS TO EVIDENCE OF INVASION (CARCINOGEN REMOVED ON DETECTION OF FIRST MALIGNANT CELLS.)

Treatment	No. of animals	No. of weeks	Mean \bar{x}	S.D. σ_{n-1}
N.H.S.	23	20; 21; 24; 21; 35; 28; 32; 31; 26; 28; 28; 26; 24; 33; 32; 30; 21; 26; 21; 23; 29; 31; 33;	27	4.59
A.L.S.	28	15; 18; 11; 20; 30; 11; 19; 17; 21; 27; 15; 23; 27; 20; 23; 13; 13; 16; 15; 18; 23; 17; 10; 26; 21; 17; 21; 15;	18.6	5.18
SALINE	22	33; 25; 29; 27; 32; 26; 25; 17; 22; 30; 23; 25; 34; 35; 28; 27; 37; 31; 22; 28; 20; 27;	27.4	5.0
B.C.G.	15	32; 27; 33; 35; 42; 24; 32; 38; 41; 34; 37; 23; 36; 42; 39;	34.3	6.0

F) Modification of the histopathology by A.L.S. and B.C.G.

Mice from two of the groups of experiments in which the carcinogen had been removed, L.T. 1 and L.T. 2, were used to study the histological patterns in the immunosuppressed and immune stimulated animals, to compare these to each other and to the control groups, and to determine if any differences could be demonstrated.

In the mice treated with A. L.S. or B.C.G. there was no alteration in the histological type of carcinoma produced, these being predominantly well-differentiated squamous carcinomas with no evidence of any increase in undifferentiated tumours in either group compared to the controls. Nor were the early types of intra-epithelial lesions altered in any way, being formed by a diffuse proliferation of the basal and parabasal cells as in the control groups. Underlying these relatively normal epithelia, however, in a few mice, occasional solitary aggregates of lymphoid cells and macrophages could be found (Fig. XXIX). These aggregates were separated from the epithelium by fibrotic connective tissue and associated with these cells were mast cells and eosinophil leucocytes. No epithelial cells, either viable or degenerate, could be identified by light microscopy in these lesions, nor could any keratin debris be found. The intra-epithelial lesions that could be classified as definitely pre-invasive, severe dysplasia and in-situ carcinoma, were histologically and cytologically similar in the experimental groups and in the controls. With this type of lesion there was, however, in the experimental groups and controls, variation in the response in the underlying tissues. In the immune competent control groups, associated with the intra-epithelial lesions there were dense lymphocyte and plasma cell infiltrates in the underlying stroma, these infiltrates being closely applied to



FIG. XXIX. Aggregates of lymphoid cells and plasma cells occasionally found underlying relatively normal epithelium.

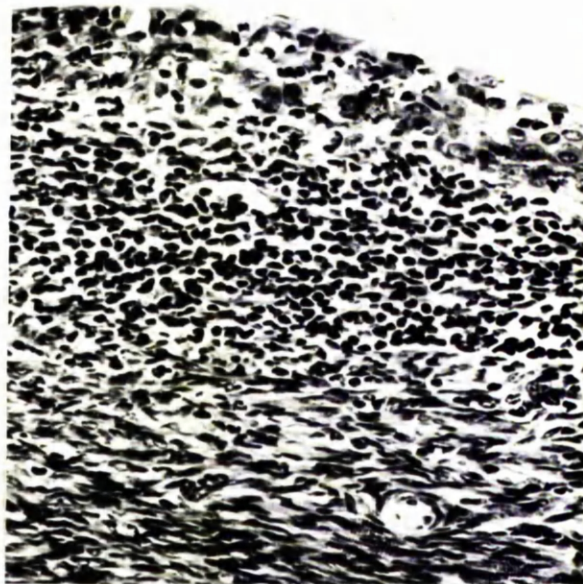


FIG. XXX. Lymphoid and macrophage infiltrate underlying intra-epithelial neoplasia.

the basement membrane. The cellular infiltrates were only apparent at the segment of epithelium which had undergone changes suggestive of neoplasia even if this was still intra-epithelial (Fig. XXX). Associated with the severely dysplastic epithelial changes, the lymphoid cells were aggregated in focal clumps while underlying the in-situ carcinoma, the cellular infiltrate tended to be more diffuse. The adjacent epithelium, normal or with only mild dysplasia, had no associated lymphoid cell aggregation.

However, in the experimental group receiving A.L.S. there was a markedly reduced chronic inflammatory response so that little difference could be found between the stroma underlying the normal or mildly dysplastic epithelium or the stroma, underlying segments of epithelium showing definite intra-epithelial malignancy. In the stroma underlying both types of epithelium there were sporadic lymphocytes and plasma cells, occasionally in small groups, but no dense cellular aggregations closely applied to the basement membrane (Fig. XXXI).

In the groups receiving B.C.G. the cellular response was more diffuse, but in these areas where there were definite intra-epithelial malignant changes, the lymphoid infiltrate was not only intense in the underlying stroma but numerous lymphoid cells could be found in the atypical epithelium (Fig. XXXII). Plasma cells were also numerous in the underlying stroma. However, the most significant changes were segmental lesions where the inflammatory infiltrate was so intense, and so involved both the stroma and the full thickness of the epithelium, that it obscured the epithelial cells with the result that none could be identified either in their normal position nor in the underlying tissue (Fig. XXXIII). In this type of lesion there were also large numbers of macrophages and the appearances suggested a vigorous response to and destruction of, either pre-invasive or early invasive malignancy.

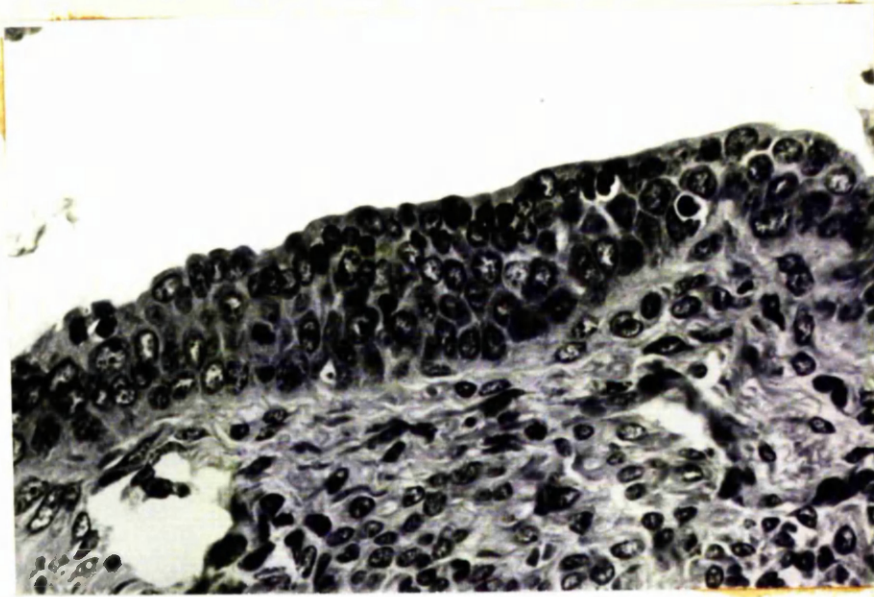


FIG. XXXI. From a mouse receiving A.L.S. : Little evidence of any lymphoid infiltrate in the stroma underlying the atypical epithelium.

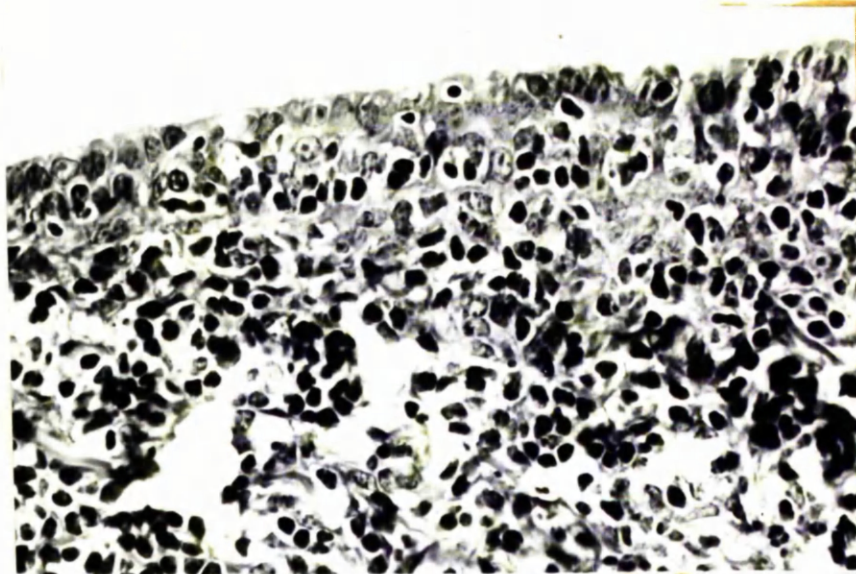


FIG. XXXII. From a mouse receiving B.C.G. : Lymphoid cells and macrophages in the stroma and in the epithelium.

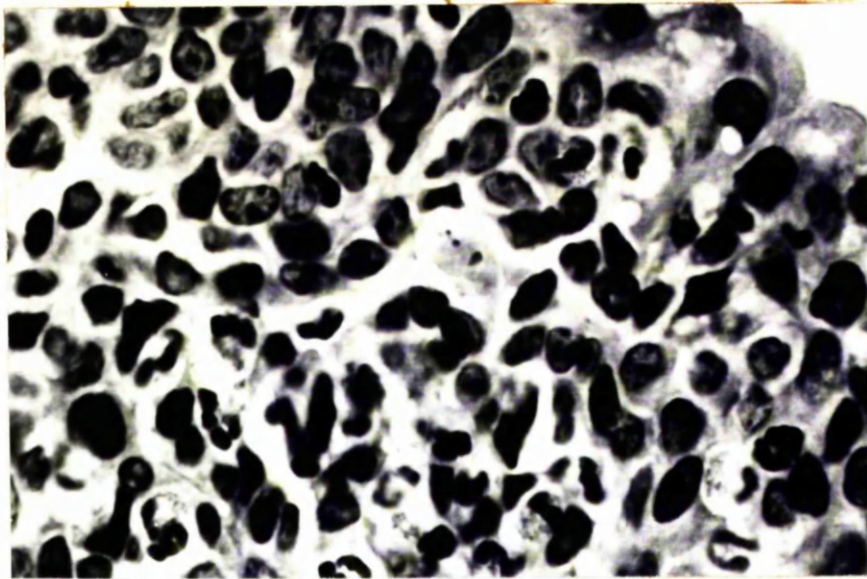


FIG. XXXIII. From a mouse receiving B.C.G. The lymphoid and macrophage cellular infiltrate has obscured the epithelium.

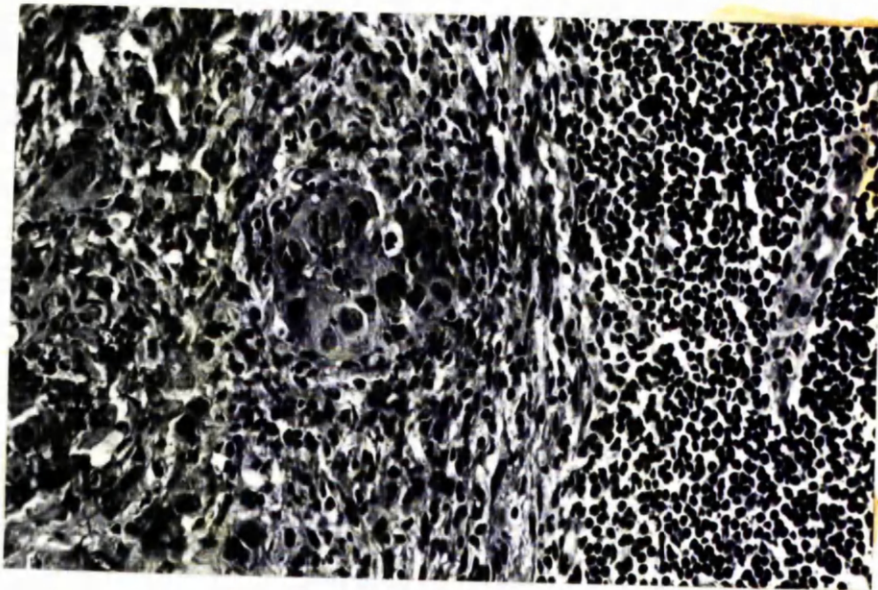


FIG. XXXIV. Micro-invasion with the nests of carcinoma cells surrounded by lymphoid cells and macrophages.

When micro-invasion and early invasion occurred in the immuno-competent controls the nests and cords of invasive carcinoma were frequently surrounded by large numbers of lymphocytes, plasma cells and macrophages (Fig.XXXIV).Fibroblasts also appeared to be increased and there was usually moderately increased fibrosis of the sub-epithelial tissues. In association with the early invasion lesions, mast cells and eosinophil leucocytes were also numerous.

In the mice receiving A.L.S., in those lesions that could be classified as showing the earliest evidence of micro-invasion there was a marked difference in the tissue response to the micro-invasion compared to either the control animals or to the groups receiving B.C.G. In this situation there was a markedly reduced, mononuclear cell response and those cells that were present were mainly plasma cells. Mast cells and eosinophils were also present in reduced numbers. The histological picture of the earliest invasion was of thin cords or finger-like projections of basal type cells invading into the stroma with little evidence of any cellular response (Fig.XXXV), but with a moderate fibrous reaction around the advancing lesions.

The earliest forms of micro-invasion in the animals receiving B.C.G. were obscured by the intensity of the inflammatory response in which lymphoid cells and macrophages predominated (Fig.XXXVI) and the invading process often included degenerating epithelial cells suggesting retardation of the invasion (Fig.XXXVII). Where the invading lesions had differentiated to form definite squamous epithelium, plasma cells were prominent and eosinophil leucocytes and mast cells were also greatly increased. Early fibrosis was also a feature of this type of development.

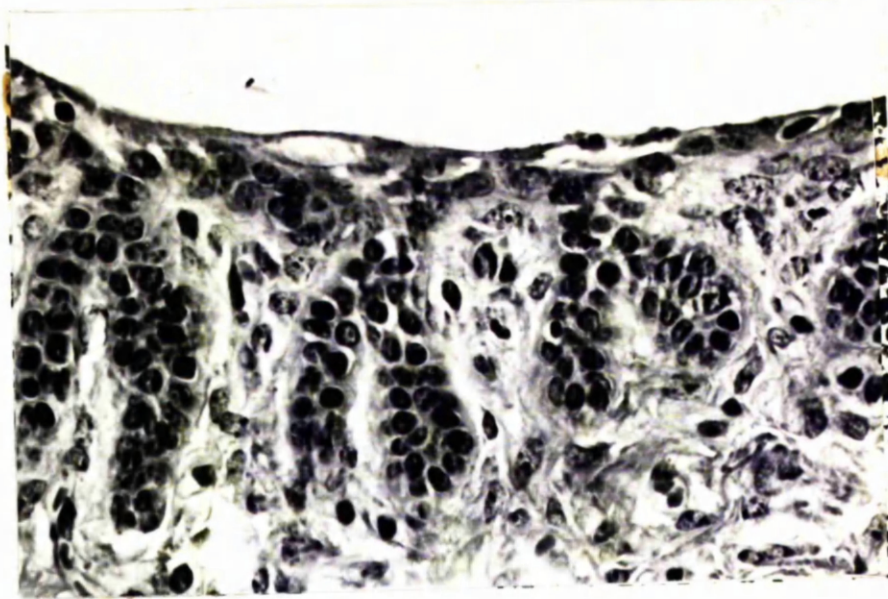


FIG. XXXV. From a mouse receiving A.L.S. : Finger-like projections of atypical basal cells invading into the underlying stroma with little evidence of cellular response.

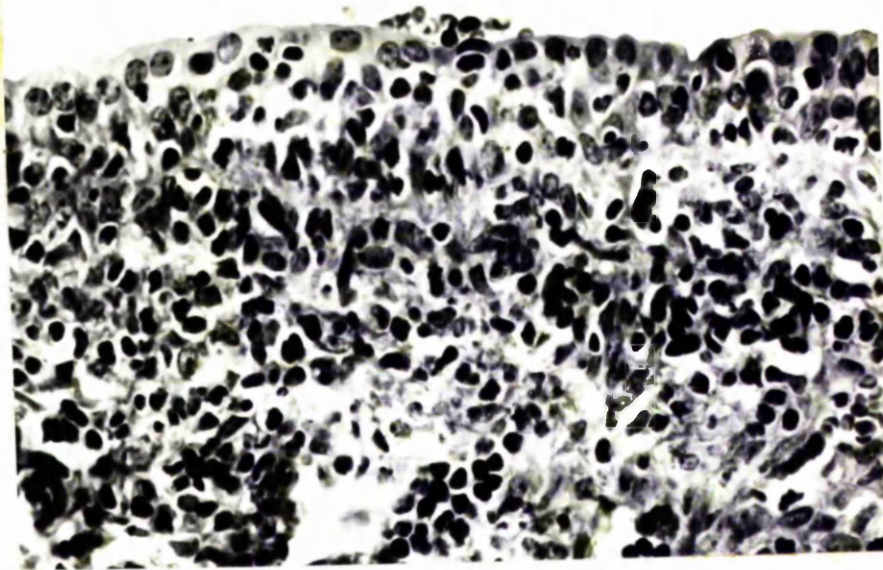


FIG. XXXVI. From a mouse receiving B.C.G. Intense lymphoid and plasma cell infiltrate obscuring the early invasive lesions.

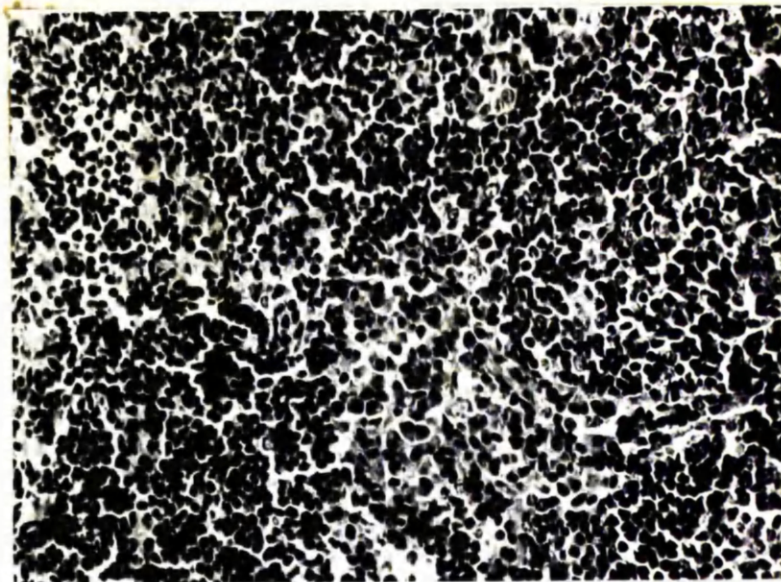


FIG. XXXVII. From a mouse receiving B.C.G. Degenerating epithelial carcinoma cells associated with an intense lymphoid and macrophage infiltrate.

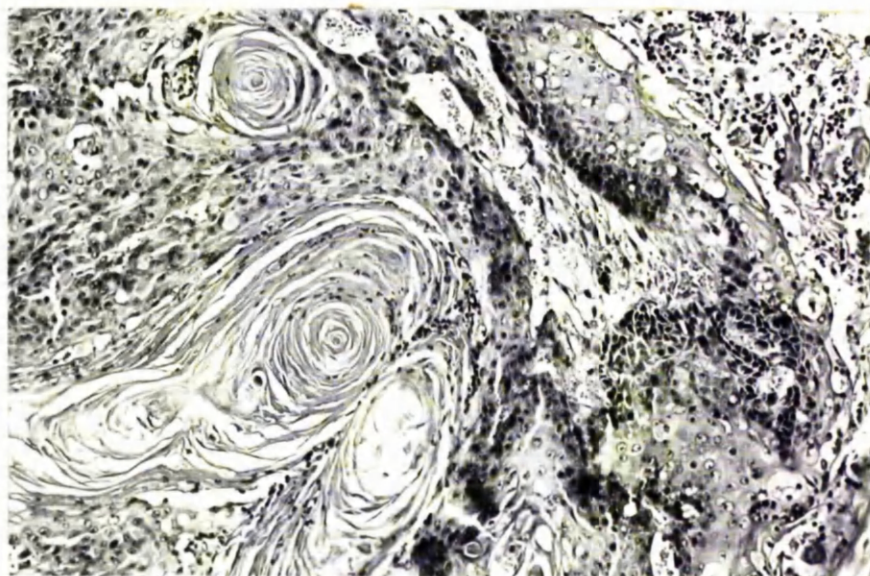


FIG. XXXVIII. Advanced invasive carcinoma with only a slight lymphocytic response at the periphery.

When definite invasion and tumour formation had occurred there appeared to be a marked reduction in the tissue responses in the control group, this being represented mainly by a comparatively slight lymphocytic response at the periphery of the carcinoma (Fig.XXXVIII).In the A.L.S. group there appeared to be even fewer lymphocytes. In the group receiving B.C.G. there was a reduced response compared to the response B.C.G. evoked in the earlier lesions, but it was increased compared to the control groups and to the A.L.S. groups with a similar sized tumour. In the B.C.G. group the inflammatory response was also mainly at the periphery of the tumour and around small separated tumour cell groups.

DISCUSSION.

If an immunological surveillance mechanism (291) is operative in the normal organism its effect should be to protect against small numbers of neoplastic cells localised as incipient clonal foci and the development of frank neoplasia would then signify the breakdown of the system either artificially by immunosuppression or by old age (292, 293).

For immunological mechanisms to inhibit the formation and growth of clones of malignant cells, the neoplastic cells must be exposed to the host's immunological apparatus and there must be sufficient antigenic dissimilarity between the neoplastic cells and the host cells to result in an immune response being mounted. A further requirement, necessary to prevent the establishment of malignant cells, is that the tumour associated antigens should be expressed in the early populations of tumour cells.

Evidence for tumour antigen expression in pre-malignant lesions is provided by studies on M.C. induced skin papillomas in which it has been established that the number of papillomas developing in skin initially treated with carcinogen and then grafted to syngeneic mice correlated with the immune competence of the recipients (294); decreased immunological competence due to sub-lethal irradiation accelerated the appearance of papillomas and increased their incidence while immune stimulation with methanol extracted residues of B.C.G. delayed the appearance of papillomas and decreased their incidence. It was also reported that the neo-antigens persisted through the progression to malignancy and were detectable in the skin carcinomas derived from the individual papillomas (31). This would appear to

invalidate the claim, derived from studies involving transplantation of malignant cells, that the immune response must be rapidly mounted to eradicate the malignant cells before they become established as tumours, since in the usual situation in the development of malignancy the antigens would be present in the relatively long pre-invasive phase, and emphasises the fact that experiments with transplanted fully malignant cells have little bearing on the immune responses against early in-situ neoplasia, since the insertion of a comparatively large number of pre-selected tumours cells into an immunologically virgin secondary host is in no way analagous to the formation and growth of a primary tumour in its unique micro-environment.

In this situation in which neo-antigens persist from benign into malignant lesions, the possibility exists that the shedding of antigenic tumour membrane products from the cells of the pre-malignant lesions may result in local conditions in which are formed antigen-antibody complexes and these might, in turn, abrogate any cell mediated response against malignant clones of cells which subsequently emerge carrying the same antigens, thus allowing invasion to take place.

It is also possible that modification of the systemic immune response by the released antigen products of the pre-malignant lesions may be such, that any malignant clones of cells that emerge carrying the same antigens may be protected from destruction, in a similar way in which kidneys transplanted into recipients who have received multiple blood transfusions are protected (295).

Further studies, investigating the influence of immune status on the progression or regression of papillomas, established that not only did a greater number of papillomas develop in M.C. treated mouse skin transplanted to immuno-suppressed

syngeneic mice than occurred in control or immune-stimulated mice, but that the immune reactivity of each group of mice correlated with the regression of the papillomas, the greatest proportion (71%) regressing in the immune-stimulated group, while the lower proportion (30%) regressed in the immune-suppressed group (296).

Papilloma progression to carcinoma in transplanted M.C. treated skin was also influenced by the immunocompetence of the host : the time of appearance of squamous cell carcinoma was shorter and the final incidence higher in immunosuppressed mice (296).

These studies established that early populations of methyl-cholanthrene transformed cells have neo-antigenic specificities capable of inducing an immunological rejection reaction, but these effects have only been detected by transplantation manipulation which probably serves to call attention to the neo-antigens.

Investigations on the effect of immunosuppression on the various stages of skin carcinogenesis, in which transplantation was not used, presented a pattern contradictory to the preceding studies. Short-term immune impairment by A.T.S. did not modify the initiating effect of topical D.M.B.A., nor the subsequent response to promotion with croton oil skin painting, as measured by tumour incidence, latent period and degree of progression to malignancy compared to normal controls (11).

Similar experiments comparing the effects of autografting M.C. treated skin with undisturbed animals suggested that while they remain undisturbed the developing lesions do not stimulate an immune response which will eliminate the developing malignant cells, questioning the contribution of immune mediated

rejection in-situ. The postulate here is that the increased vascularity associated with the inflammatory response to the trauma of transplantation increases the numbers of lymphocytes passing through the tissues and brings these into contact with the neo-antigens in the developing malignant cells thus initiating an immune response, and that in the undisturbed lesion this does not happen (147).

It is, however, equally possible that the inflammatory response associated with transplantation disrupts the micro-environment in which the in-situ lesion is developing and that if this contains free antigen or antigen-antibody complexes, which are blocking the cell mediated response to the incipient neoplasm, then disruption of this micro-environment may cause dilution of the complexes by oedema, their removal by the increased flow of tissue fluids, or alteration in the antigen-antibody ratio by macrophage or polymorphonuclear leukocyte digestion of the antigen or the complexes; by whatever means, the loss of the free antigen or complexes may allow the lymphocytes to destroy the neoplastic cells.

This explanation of the course of events is in many ways more tenable than the concept that in-situ lesions do not evoke a response and thus lymphocytes do not gain access to the lesion to be stimulated, since the histological examination of in-situ lesions often demonstrates an intense lymphoid exudate closely applied to the lesion (24, 27). It should be noted, however, that in common with the lymphoid response there is also a marked plasma cell response with the presumed production of antibody capable of blocking cell mediated responses.

Similar considerations may apply to M.C. induced hyperplastic breast nodules which are immunogenic and are rejected on transplantation to syngeneic mice but which appear to arouse little reaction while in-situ in the mammary pad (297). A complication here, however, is that it is claimed that the mammary pad is an immunologically privileged site (298).

Contradictory results are reported in two experiments investigating the effect of A.L.S. on the development of pre-malignant liver lesions (13, 153). In the first experiment (13) the incidence of pre-neoplastic lesions was increased whereas in the second (153) no difference in number or size of the precancerous foci could be detected. However, in this second group, in one experiment in which animals were treated with A.L.S. to the stage where precancerous lesions should have been present, and then left untreated, there was a much higher incidence of tumours over the controls.

In our experiments in which the carcinogen remained in position we failed to demonstrate any difference in the length of time to the detection of the first malignant cells and to the time of formation of either severe dysplasia or in-situ carcinoma in either the immune stimulated or immune suppressed animals when the groups were compared to each other or to the controls. These findings are in agreement with the experiments in which papillomata are allowed to develop undisturbed and in which no evidence of any effect of alteration of the immune response could be demonstrated (147).

Nor could we find any difference in the length of time for the progression of severe dysplasia or in-situ carcinoma to invasive carcinoma, associated with either immune stimulation with B.C.G. or immune suppression by A.L.S. These

experiments were also in agreement with the experiments in skin carcinogenesis in which the lesions are allowed to progress undisturbed.

The apparent finding of an increased number of tumours in the A.L.S. group of the S.T. experiments (Table I) compared to the B.C.G. group, and the controls, suggests an effect of immune suppression or immune stimulation, but if the number of micro-invasive carcinomas is added to the number of tumours, the total number of invasive lesions in all groups becomes almost equal and suggests a modest acceleration of tumour growth by A.L.S. and tumour retardation by B.C.G., which is probably exaggerated by the short length of the experiment. This would be in agreement with those experiments in which the carcinogen is injected and in which only a modest increase in the latent period could be demonstrated, the ultimate tumour numbers being the same no matter what immunological manipulations were used.

This point is emphasised by the finding that in the S.T. experiment there was no significant difference between the various groups in the number of mice in which the cervical epithelium returned to normal. At the termination of the experiments virtually no animals had normal epithelium, meaning that in these animals there is a continuing potential for the development of carcinoma and provides a possible explanation for those experiments in which the injection of relatively large amounts of carcinogen ultimately gave rise to the same number of tumours, in spite of immune stimulation, if the experiments are continued long enough : it is that the malignant potential of the transformed cells outlasts the immune stimulated state and as this wanes outgrowth of the invasive lesion occurs.

In these experiments, however, the influence of the continuing presence of the carcinogen raises several questions.

The immunosuppressive action of methyl cholanthrene on humoral antibody has been established (299, 300) and it has been substantiated that the carcinogenic dose is sufficient to depress immune responses (300) and that the effect of a single dose resulted in prolonged immunosuppression over a period of several months (301). Administration of small doses, either by subcutaneous injection or skin painting led to a cumulative effect. (131). Evidence that methyl cholanthrene also exerts a suppressive effect on cell mediated immunity, was provided in studies demonstrating increased survival of male to female skin grafts and accelerated growth of first transplant generation sarcomas in treated mice (302, 303).

Interference with the cellular rather than the humoral responses is more likely to be a significant factor in modifying carcinogenesis and a correlation between methyl cholanthrene induced cellular immunosuppression and its carcinogenic effect has been demonstrated (303).

These effects have all been demonstrated systematically using serum and circulating lymphocytes but little is known about the local effects of the carcinogen. Even when carcinogen administration does not lead to systemic immunosuppression a local effect on the immune responses may prevent the destruction of nascent tumour cells in the area of carcinogen application. In this respect, it is noteworthy that it has been shown that subcutaneous tissue transplanted from the site of injection of benz (a) anthracene or di benzo (ai) pyrene accelerates epidermal carcinogenesis. If a phenomenon such as local

Immunosuppression actually occurs during chemical carcinogenesis then the addition of a systemic immunosuppressive might not be expected to alter markedly the degree of tumour induction. Similarly generalised B.C.G. immune stimulation might not overcome local immunosuppression although the ability of B.C.G. to overcome systemic carcinogen-induced immunosuppression has been demonstrated (304).

The immunosuppressive component of chemical carcinogenesis may explain why the effects of thymectomy and A.L.S. immunosuppression are no more dramatic in increasing tumour incidence : this probably depends on the differential between the immunosuppressive action of the chemical carcinogen and the residual rejection potential after thymectomy or A.L.S. treatment.

It should be noted, however, that the same dose of M.C.A. can have a markedly different carcinogenic effect in different strains of mice (139) probably due to genetic differences in the susceptibility to carcinogenesis by this chemical, and that the demonstrable effect of A.L.S. in increasing tumour incidence is inversely related to the susceptibility of the strain of mice to M.C.A. In strain I mice, in which M.C.A. produces limited numbers of tumours, A.L.S. will greatly increase the incidence of tumours, whereas in C₃H strain, in which the same dose of M.C.A. will produce tumours in virtually 100% of mice, any effect of A.L.S. will be lost in the overwhelming effect of M.C.A. In experiments in C₃H mice, using a technique for M.C.A. induction of carcinoma of cervix similar to ours, and following the progress with exfoliative cytology, the rate of malignant transformation was so rapid that in the short time taken to

process and examine the cytology specimen used in the detection of the first malignant cells, and killing the animal (< 24 hours) invasion occurred and could be recognised in the post mortem specimen. Obviously in this type of situation no effect of systemic immunosuppression on carcinoma formation could be demonstrated (28). In Swiss mice such as are used in our experiments a long pre-malignant phase can be recognised (305).

Another demonstration of the effect of A.L.S. in a situation where a limited number of tumours develops is given in a single strain of mice (C57BL/6 x BALB/c) F1 in which increased tumour production with A.L.S. was detected in the group receiving a low dose of M.C.A., compared to controls receiving the same dose (140).

These results suggest that A.L.S. might potentiate tumour production only under these circumstances where a limited oncogenic effect of M.C.A. is demonstrated.

In most studies the effect of B.C.G. on chemical carcinogenesis has been temporary(8,286)and in several of the more encouraging studies an apparent decrease in tumour incidence may be due to a delay in the appearance of tumours that would have developed, if the life of the animal had not been cut short by the short duration of the experiment (272). This would be in agreement with our S.T. experiments in which at the termination of the experiment, despite B.C.G. immune stimulation, the epithelium was still of a type - severe dysplasia or in-situ carcinoma - with the potential to produce invasive carcinoma.

Many studies on the effect of B.C.G. on chemical carcinogenesis have been performed with high doses of chemical carcinogenesis capable of producing

tumours in 100% of animals in a short time. It is possible with the high dosage of carcinogen used in these experiments, that sufficient carcinogen to induce neoplasia may remain unmetabolised at the site of infection, after the immunostimulatory effects of B.C.G. have ceased. If the immunosuppressive effect of chemical carcinogenesis is of any significance then high doses of these carcinogens could both induce the formation of malignant clones and prevent their elimination. Although it is known that B.C.G. can reverse the immunosuppressive effects of certain carcinogens it is not known how long reversal lasts, since all the experiments demonstrating the effect were of short duration (304), and with the high doses used in some experiments it is possible that some immunosuppressive effect may remain after the B.C.G. stimulation has lessened. It is also not known if systemic B.C.G. can overcome any local immunosuppressive effects of a high concentration of carcinogen, although if injected admixed with the carcinogen a marked decrease in the incidence of tumours resulted (306), suggesting that local events were more important than the systemic events. Similar effects have been demonstrated with intramuscular injection of an admixture of B.C.G. with the mouse sarcoma virus of Maloney (MSV - M) which suppressed the development of the tumour at the injection site (307). Protection was increased if the animals were previously immunised with B.C.G. In this situation, however, although the immune response may aid the host defence against neoplasia, it is possible that B.C.G. might act by limiting the infection and persistence of the oncogenic virus, since animals infected with B.C.G. resist viral infections (8,308), and the intravenous injection of tuberculin can stimulate the release of interferon in B.C.G. immunised mice.

A situation analogous to this might exist with regards to the chemical carcinogenesis since it is known that macrophages and monocytes possess at least one enzyme that catalyses the metabolism of polycyclic hydrocarbon carcinogens (309). By increasing the enzyme levels within these cells B.C.G. stimulation could greatly accelerate the active conjugation or excretion of these compounds so reducing the carcinogenic potential, and no immunological processes need necessarily be involved.

The experiments of Lappe (294) avoided the effects of systemic immunosuppression by transplanting the methyl cholanthrene treated skin of syngeneic recipients. Injection of methanol extraction residues of B.C.G. delayed the appearance of macroscopic papillomas and decreased their incidence. Histological examination revealed a number of sub-macroscopic papillomas in excess of the controls, and many of these papillomas were associated with localised lymphocytic infiltrates. The incidence of carcinomas could be correlated with the total number of days that mice bore papillomas suggesting that prevention of carcinoma formation depended on the elimination of pre-malignant lesions. In this type of experiment, however, the trauma associated with transplantation, adds its own problems to the difficulties of interpreting events.

In an attempt to reduce the effects associated with the continuing presence of the carcinogen in our second group of experiments, the L.T. group, we removed the carcinogen and to avoid the problems associated with short term experiments we allowed these experiments to continue for one year after the implantation of the carcinogen.

In these experiments a divergent effect of A.L.S. and B.C.G. can be seen. In the L.T. group I there was a significant difference in the number of mice developing tumours between the A.L.S. group, 12 out of 18 mice, 66%, and the group receiving B.C.G. 6 out of 21 mice, 28.5%. In contrast to the S.T. experiments, however, as the stages of carcinogenesis are added together then the disparity becomes more marked. When the numbers of mice with micro-invasive carcinoma are included it can be seen that the difference in the numbers of mice developing invasive lesions, between the groups receiving A.L.S., 15 out of 18 mice, 83%, and the group receiving B.C.G., 7 out of 21 mice, 33%, becomes more marked while in the S.T. group the difference became less marked. This contrasting effect of A.L.S. and B.C.G. when the carcinogen had been removed, is emphasised when the number of mice with all invasive or potentially invasive lesions are included at the termination of the experiment. It can be seen that 17 out of 18 mice, 94%, receiving A.L.S. had either developed, or had the potential to develop, invasive carcinoma, whereas in the group receiving B.C.G. only 10 out of 21 mice, 47.5%, were in this category. The control groups were intermediate to the two experimental groups. This contrasts also with the S.T. experimental groups in which, at the termination of the experiment, the numbers of invasive or potentially invasive lesions were virtually identical in all groups, experimental or control.

Further emphasis for the divergent effects of A.L.S. and B.C.G. in the L.T. experiments are found in group L.T. I in the figures for the numbers of cervixes which had returned to normal at the termination of the experiment ; in the A.L.S. group only 1 out of 18 mice had an apparently normal cervix, whereas

in the B.C.G. group 9 out of 21 mice had reverted to normal. In the control groups, 4 out of 16 mice, 25%, and 4 out of 18 mice, 22%, had returned to normal from a potentially invasive epithelium.

In L.T. group 2, in which A.L.S. and B.C.G. were started on the day of removal of the carcinogen, the figures for all the groups were similar to those of L.T. 1, Tables V&VI, and no effect could be demonstrated, of the delay in giving A.L.S. or B.C.G.

The experiments L.T. 1 and L.T. 2 demonstrate that when the influence of the carcinogen is removed, A.L.S. or B.C.G. have a marked effect on the progression or regression of potentially invasive epithelium at normal tissue interfaces. In this situation A.L.S. prevents the reversion of the epithelium to normal and promotes the development of invasive carcinoma, while B.C.G. increases the number of cervixes reverting to normal in comparison to the controls and the A.L.S. group, and decreased the number of carcinomas forming. This is also in contrast with the situation in the S.T. experiments where at the termination of the experiment in the animals receiving B.C.G. there was little evidence of any reversion to normal.

In L.T. group 3 and 4 an attempt was made to further reduce the influence of the carcinogen by waiting three months after removal of the carcinogen and sub-dividing the mice into two experimental groups, L.T. group 3 with cytological evidence of in-situ malignancy and L.T. group 4 with cytological evidence of early invasive carcinoma but with no visible or palpable tumour.

In L.T. group 3 the results, although similar to those of groups L.T. 1 and L.T. 2, were more divergent, and demonstrated a marked effect of A.L.S., by the increased incidence of tumours, the maintenance of in-situ malignancy and

a corresponding failure of the reversal of the epithelium to normal, with none of the mice receiving A.L.S. having a normal epithelium at the termination of the experiment.

Of the animals receiving B.C.G. only 3 out of 16 mice, 18.7%, formed tumours and 10 out of 16 mice, 62.5%, reverted to a normal epithelium, while in the control groups receiving N.H.S., 3 out of 20 mice, 15%, or saline, 4 out of 19 mice, 21%, reverted to normal.

In L.T. group 4 the effect of immune stimulation and immunosuppression on early invasive carcinoma was demonstrated. As might have been expected, the incidence of tumours developing in the control groups was higher than in the other L.T. groups being 60% and 62.5% in the N.H.S. and saline groups, respectively.

In the experimental group receiving A.L.S. 13 out of 15 mice, 86.6%, ultimately developed tumours and 1 out of 15 mice, 6.6%, had a micro-invasive carcinoma. One mouse had an in-situ pre-invasive lesion.

Of the group receiving B.C.G., 2 out of 15 mice, developed tumours, and 3 out of 15 had micro-invasive carcinomas, giving an incidence of 5 out of 15 mice with definitely invasive lesions. 3 out of 15 mice had either severe dysplasia or in-situ carcinoma but the most interesting observation was that, even when starting with an early invasive lesion, 7 out of 15 mice had at the termination of the experiment either mild dysplasia or almost normal cervical epithelium. This suggests that even with early invasive carcinoma occurring at natural epithelial-stomal junctions, B.C.G. could be effective in preventing tumour development and could cause reversal of the malignant epithelium to almost normal. In this group none of the control mice reverted to normal, although some did appear to regress to an in-situ stage of malignancy.

In view of the divergent effects of A.L.S. and B.C.G. on potentially invasive epithelium in the L.T. experiments, and since in the N.H.S. control groups in L.T.1 and L.T.2, 4 out of 16 mice and 3 out of 20 mice, in these groups respectively reverted to normal, and in the same groups 4 out of 18 mice and 4 out of 19 mice receiving saline reverted to normal, it is suggested that this elimination of the potentially invasive carcinoma with reversal to normal are due to immunological processes occurring in the unstimulated mice with undisturbed pre malignant lesions. This is in contrast to other experimental findings (11, 147).

Treatment of mice with B.C.G. (8, 272) or Freund's adjuvant (310) has been shown to result in some inhibition of tumour induction and to delay the appearance of tumours although not apparently affecting the final incidence.

Immunosuppression by thymectomy (125), A.L.S. (23, 129) and azathioprine (311), has been shown to increase the incidence of tumours in mice, and reduce the latent period.

It has been suggested (8) that B.C.G. given at the onset of carcinogenesis inhibits the outgrowth of the tumour but is without effect on the early processes. No evidence, however is given for this statement except that the latent period, to the discovery of a palpable tumour, is prolonged. This might be due to the selection for slow growing weakly immunogenic tumours rather than the inhibition of all tumours, or to the prevention of formation of or elimination of the early stages of neoplasia. On the other hand all the forms of immunosuppression used, thymectomy, A.L.S. or azathioprine have shortened the latent period to tumour formation but have not always increased the

incidence of tumours. No information is available on the effects of A.L.S. or B.C.G. on the stages preceding the detection of a palpable tumour.

A.L.S. or B.C.G. could produce the above effects of the latent period to the production of a palpable tumour by acting at the stage of the production of the first malignant cells, by acting on the stage of in-situ malignancy, or by acting at the later stages of invasion or tumour growth.

In the studies in the L.T. experiments of the effects of A.L.S. on B.C.G. on the length of time from the implantation of the carcinogen to the detection of evidence of invasion the results, table XI show that, in contrast to the S.T. experiments, A.L.S. considerably shortened this time while B.C.G. increased the length of this latent period.

Similar results were obtained when the length of time from the first detection of malignant cells to the time of invasion were investigated (table XIII). Here again A.L.S. shortened, and B.C.G. lengthened, the pre-invasive stage compared to the controls and to each other.

These results suggest that A.L.S. and B.C.G. have some effect on the early stages of neoplasia and invasion and do not merely act in the later stages of tumour formation. Unfortunately we found it impossible to devise an experiment, using this model, which would allow us to implant the carcinogen for the minimum time that would result in a consistent yield of malignant changes, while allowing the removal of the carcinogen at some stage before the formation of malignant cells. This might then have allowed some insight into the effects of A.L.S. or B.C.G. on the length of time to the emergence of the first malignant cells without the continuing effect of the carcinogen. In our attempts at achieving

this state no reliable indicator of any very early stage in the neoplastic process at which the carcinogen could be removed resulting in a consistent yield of tumours, to which the A.L.S. group or B.C.G. group could be compared was obtained. Nor did the implantation of the carcinogen for a short fixed time give the necessary reproducible tumour yield although numerous different short periods of implantation were tried. This would have been the ideal test for the effect of A.L.S. or B.C.G. on early carcinogenesis and the emergence of clones of malignant cells.

Conflicting observations of the efficiency of B.C.G. in preventing tumour development and of A.L.S. in facilitating tumour formation may depend on different schedules of administration.

Pretreatment with B.C.G. has been effective in some studies though not in others, and a protective effect has been observed when B.C.G. has been given before or at the time of exposure to the carcinogen, or after exposure to the carcinogen but before the appearance of a visible tumour. A significant delay in the appearance of fibrosarcomas was recorded (286) when B.C.G. was given to mice either 2 weeks before or 2 weeks after methyl cholanthrene injection, but if the B.C.G. was delayed until 50 days after the methyl cholanthrene, at which time the first tumours were detectable, the appearance of fibrosarcomas in the remaining animals was accelerated. B.C.G., the same day as the carcinogen, dimethylbenz (a) anthracene, by mouth, delayed the appearance of mammary carcinomas, but if the B.C.G. was given to rats already bearing primary tumours the appearance of secondary

tumours was accelerated (287).

Similar effects have been found with variations in the timing of A.L.S.¹ relative to the carcinogen (11 , 150).

In our S.T. experiments no effect of the variation of the timing of the A.L.S. or B.C.G. administration at the various stages of carcinogenesis could be demonstrated. In the L.T. experiments 1 and 2 again there was no significant difference in the numbers of tumours developing, nor the numbers of early invasive or pre invasive lesions when the different forms of treatment were compared to their counterpart in the other groups.

In the L.T.3 experiment in which the A.L.S. or B.C.G. were given three months after the removal of the carcinogen the effect of A.L.S. was slightly more marked than in the L.T.1 and 2 experiments as was the effect of B.C.G. when compared to the similar groups in experiments L.T.1 and 2.

In the experiment L.T.4, when A.L.S. or B.C.G. were given to early invasive carcinomas, A.L.S. greatly accelerated tumour formation and B.C.G. caused reversal of the early invasion.

This suggests that although immune responses may be active in the early stages of neoplastic transformation that the maximum systemic response may occur immediately the basement membrane breached and invasion into the underlying stroma occurs. If the B.C.G. or A.L.S. is given at this time, to augment or depress the natural response then maximum effects might be obtained.

Histological examination of the tissues provides some histopathological basis for the preceding observations.

In the stroma underlying the pre cancerous and early cancerous lesions there was an intense cellular stromal reaction in the groups receiving B.C.G. compared to the groups receiving A.L.S. where the response was greatly reduced.

This disparity was even more marked in the stages of early invasion where the groups of invasive cells, in the B.C.G. stimulated animals were frequently surrounded by masses of lymphocytes and macrophages, contrasting sharply with the A.L.S. suppressed animals in which finger like projections of invasive cells often appeared to evoke no stromal response, or a very reduced response.

When tumour formation occurred then in both groups the stromal response was similar being often reduced to small aggregates of small lymphocytes.

CONCLUSIONS

In experiments on the effects of immune stimulation and immune suppression in chemical carcinogenesis, account must be taken of the continuing presence of the carcinogen. Divergent effects of immunosuppression and immune stimulation, where any effects of the chemical carcinogen are reduced, can be demonstrated.

Whether the main effect of the carcinogen is local or systemic is arguable but our experiments would suggest that local events are paramount and that against these the alterations in the systemic immune responses are of relatively little consequence.

Our findings also suggest that where the effect of the carcinogen is removed then alterations in the immune status of the host will affect the early neoplastic processes, especially the duration of the in situ malignancies, as well as the later stages of tumorigenesis, although the effect of B.C.G. on micro invasive carcinoma suggests that a maximal host response may naturally be occurring at this stage. This is also supported by histological studies where the cellular response is most intense around the B.C.G. stimulated mice with micro invasive lesions.

We disagree with those findings which suggest that intra epithelial malignancies do not evoke an immune response capable of retarding its progression and growth, or of mediating its regression, and provide an alternative interpretation of the events responsible for the rejection of transplanted in situ malignancies. Again our findings appear to be supported by histopathological findings.

Our demonstration, at the termination of S.T. experiments, of the presence of epithelium with the potential to produce invasive carcinoma provide a basis for the results obtained in other experiments on chemical carcinogenesis of short duration, where immune stimulation delayed but did not prevent tumour formation.

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