1 INTRODUCTION
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A cell, of any origin has a definite regulatory mechanism for its existence. In animals, a balance is generally maintained between cell renewal and cell death in most organs and tissues. The various types of mature cells in the body have a given life span; as these cells die, new cells are generated by the proliferation and differentiation of various types of stem cells. Under normal circumstances, the production of new cells is so regulated that the numbers of any particular type of cell remain constant. Occasionally, though, cells arise that are no longer responsive to normal growth-control mechanisms. These cells gives rise to clones of cells that can expand to a considerable size producing a tumor, or neoplasm.

A tumor, which is not capable of inducing growth and does not envade the healthy surrounding tissue extensively, is benign. A tumor that continues to grow and becomes progressively invasive is malignant; the term cancer refers specifically to a malignant tumor. In addition to uncontrolled growth, malignant tumors exhibit metastasis; in this process, small clusters of cancerous cells dislodge from a tumor, invade the blood or lymphatic vessels, and are carried to other tissues, where they continue to proliferate. In this way a primary tumor at one site can give rise to a secondary tumor at another site.

Malignant tumors are classified according to the embryonic origin of the tissue from which the tumor is derived. Carcinomas are tumors arising from endodermal or ectodermal tissues such as skin or the epithelial lining of internal organs and glands. Sarcomas, which arise less frequently, are derived from mesodermal connective tissues such as bone, fat and cartilage. The leukemias and lymphomas are malignant tumors of haematopoietic cells of the bone marrow. Leukemias proliferate as single cells, whereas lymphomas tend to grow as tumor masses. It is important to realize that cancer is not a single disease with a single cause and a single type of treatment. There are more than 200 different kinds of cancer, each with its own
name and treatment. The major forms of treatment of cancer are chemotherapy or radiotherapy. Therefore, it is highly desirable to identify methods by which selective killing of cancer cells is increased while preventing the side effects (65).

1.1 CANCER THERAPY

1.1.1 RADIOThERAPY OF CANCER

Radiotherapy plays an important role in the treatment of cancer. Cancer cells are more sensitive to radiotherapy than normal cells and will, therefore, be destroyed at a greater rate. Radiotherapy stops the growth of cells by interfering with their normal function. The normal cells in the area being treated will also be affected but, unlike cancer cells, they are able to repair themselves very quickly. Irradiation is popular because it does not require any cutting or incision and therefore has no immediate mechanical effects on the function of organs. It does, however, damage the living cells. It causes mutation that is also a cause for the development of cancer. Radiotherapy has a stronger damaging effect on cancer cells than it has on normal cells, particularly if its administration has been calibrated at a favorable energy level. It is therefore regarded as an effective anti-cancer treatment.

Radiotherapy is generally applied in combination with surgery and chemotherapy; its use as the sole agent for treatment is rather rare. The object of its combination with other modalities is to supplement the effects of the other therapies.

The following are the options in the administration of radiotherapy that depend on the course of treatment and location of the malignancy:

1. Irradiation of the external body,

2. Irradiation through a device inserted into a body cavity or
3. Irradiation of the tumor in which there is direct contact between the radiation source and the cancer cells

Ionizing radiation - similar to x-rays - can penetrate tissue, and alter the part of the cell, which regulates its growth and reproduction. Uncancerous cells can recover from this damage, while cancer cells cannot. There are two types of radiotherapy - delivered from outside the body by a machine, and using radioactive implants placed inside the body. Researchers are working to increase the effectiveness of radiotherapy by targeting the beam of energy more precisely, and making the cancer cells more sensitive to it.

The principal use of radiotherapy is to tackle solid tumours found in just one location, for example skin, brain, breast or uterine cancers. Sometimes doctors will use the treatment to shrink a tumour so that a subsequent operation will be more effective. In some cases, for example in invasive bladder cancer, radiotherapy is considered as the first option, as an alternative to surgery that would have permanent effects on the lifestyle of the patient. But although radiotherapy alone can cure many cancers, in other cases the radiotherapy is given after surgery over the surrounding area to "mop up" any remaining cells which have spread from the original cancer site. If there is a suspicion or firm evidence that cells could have spread further afield, then chemotherapy may be the preferred option. Although the treatment itself is painless at the time, the cumulative effect of many sessions does produce side effects. The radiation can produce a sunburn-like effect on the skin as it passes through. The extent of this depends on the intensity and number of treatments. There can be hair loss in the area being treated, which is usually temporary. The treatment can also leave the patient feeling fatigued and generally lethargic. Ionizing radiation produces changes within the genetic structure of the body's cells, and there is a small risk that an increased radiation dose leads to changes in healthy cells, which can cause cancer. However, the dose is lessening steadily as modern radiotherapy equipment targets tumours more precisely. The risks of medical radiation exposure are
miniscule when compared to the risks to the patient's health of not having the treatment. Old-fashioned radiotherapy equipment tends to give the patient a slightly higher dose. Therefore, the main efforts are now focus on delivering a more powerful radiotherapy beam accurately to smaller and smaller targets. Another field of research is looking at heating cells in a specific area to make them more sensitive to radiotherapy (36)

A great change has occurred in both medical and patient attitudes to radiotherapy. Originally regarded as a palliative treatment for inoperable or incurable cancers, it is now seen as a partner and partial replacement for surgery in the curative treatment of certain cancers such as those in skin, larynx, and cervix. Advances in both technology and technique have meant that radiotherapy can be accurately applied to the region of the cancer with minimal damage to surrounding tissue, especially the skin. How radiation kills cancer cells selectively is not fully understood but all dividing cells are particularly sensitive to radiation damage and consequently rapidly proliferating tumour cells are especially vulnerable. One of the drawbacks of radiation treatment is that therapeutic doses may also kill dividing cells in normal tissues.

Two main techniques are used for the delivery of radiation, which is given either as an external beam or as short-range radiation from an implanted radioactive source. External beam radiation usually involves megavoltage produced by linear accelerator as photons or electrons or from cobalt sources in the form of relative low energy X-rays or gamma rays. The latter are often used to treat relatively superficial lesions such as basal cell carcinoma or recurrences within the skin. High-energy radiation can be used to treat deeply located lesions such as prostatic carcinomas without delivering an excessive dose to adjacent normal tissues. The total dose of radiation is usually delivered in several fractions to maximize the tumor killing while sparing normal tissues, the exact method of fractionation differing from institution to institution.
Interstitial (implant) irradiation gives a high local dose to the tumor and usually employs sources such as radium, iridium, or caesium used in the form of needles or wires implanted in the tumor. This technique is widely used in the treatment of head and neck cancers to deliver a high tumor dose without irradiation to sensitive organs such as the lens of the eye or the spinal cord. A combination of interstitial and external radiation may be used to treat the potentially malignant peritumoural field as well as the site of the primary carcinoma. Insertion of the radioactive sources may be carried out under general anesthesia. As an alternative, tubes to contain the radioactive sources may be inserted under anesthesia and, after measurements of the implant characteristics, loading of appropriate doses of isotopes can be carried out subsequently (afterloading). This technique of afterloading has been used with caesium sources to treat cancers of the body of the uterus.

1.1.2. CHEMOTHERAPY OF CANCER

Chemotherapy is a relatively new method of treating cancer (78). The tradition of treating cancer with surgery had been in existence for over a century, and radiotherapy had been used for at least a quarter of a century before chemotherapy made its appearance in the middle of the Second World War. Malignant disease, such as leukemia, which was generalized when it first presented, or tumors, which had become disseminated, was incurable up to that time. Chemotherapy gave a first promise of cure.

Chemotherapeutic drugs act directly on tumor cells whereas other drugs must be activated by metabolic processes, either in the tumor cells or in the organs such as the liver. Some important drugs designed for cancer treatment are listed below.

<table>
<thead>
<tr>
<th>Anti-cancer drugs</th>
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</thead>
<tbody>
<tr>
<td><strong>Classification</strong></td>
</tr>
<tr>
<td>Alkalating agents</td>
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<td></td>
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</tbody>
</table>
These drugs can be divided into those that are active on dividing cells and affect a very particular phase of cell cycle (phase specific drugs), and those that affect all or most of the phases of the cell cycle. The phase and cycle specific drugs are listed as follows:

<table>
<thead>
<tr>
<th>Phase specificity</th>
<th>Acts on</th>
<th>Drugs</th>
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<tbody>
<tr>
<td>S phase specific</td>
<td>DNA synthesis</td>
<td>Methotrexate</td>
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Whatever the mode of action of the chemotherapeutic agent, a very important finding is that it destroys malignant cells according to first order kinetics; in other words the same proportion of cells is killed for each dose of the agent.

Chemotherapeutic drugs kill cancer cells. Different cancer cells respond to different drugs. Sometimes a combination of as many as eight different drugs is used in combination to get the best effect. Chemotherapy is often associated with debilitating side effects, but many types of modern chemotherapy cause only mild problems. Because chemotherapy drugs are usually injected into the blood, they travel around the body and can attack cancer cells regardless of where they find them. For this reason, doctors will use them when they think there might be cancer cells in more than one part of the body.

Radiotherapy, which uses radiation to destroy cancer cells, can only be given to small areas of the body or it will cause damage to too many healthy cells. Often, after an operation to remove cancer, chemotherapy will be given to "mop up" any remaining cells. Some cancers, such as leukemia, need chemotherapy because they involve cells, which are found throughout
Chemotherapy can be given to shrink a tumour to make it easier for the surgeon to remove. It can also ease the symptoms of patients whose cancer is not curable.

Chemotherapy, in its traditional sense, is a chemical, which is poisonous to cancer cells and kills them. This is called a cytotoxic chemical: One very early chemotherapy was produced from mustard gas, which was used as chemical weaponry during the First World War. However, anything, which is poisonous to cancer cells, may also be poisonous to the body's healthy cells, which it needs to survive. The trick with chemotherapy is to find a chemical which kills as many cancer cells as possible, and as few healthy cells. Doctors have been getting increasingly successful at developing such chemicals, by spotting the differences between the cancer cells and neighboring normal cells, and exploiting them. The principle difference between many cancer cells and normal cells is the speed at which they reproduce, or divide. Cancers tend to be dividing and growing faster than other cells in the body, which is why lumps or tumours sometimes appear. Other cancer cells may become more or less active in response to natural chemicals called hormones produced by the body. Some chemotherapy harnesses this reaction to control the growth of the cancer cells. Cancer cells are not attacked by the body's own immune defense system because the immune system does not recognize them as foreign. Some chemotherapies try to program the immune system to see the cancer cells as foreign so they can be attacked and destroyed. Normally chemotherapy is delivered by injection into a blood vein. In many cases a saline drip will be set up to dilute the drug as it enters the body. This stops to harming the vein because it is so concentrated. Sometimes, a concentrated dose of chemotherapy is needed on a particular part of the body, and side effects can be lessened by injecting it directly onto the cancerous area. For example, for some bladder cancers, the drug can be pumped into the bladder so it works directly on the tissue involved. How long chemotherapy courses last varies between different cancer types, with some being given intensively over a fortnight, normally in hospital, and some over a period of months. What side-effects can be expected? Because some chemotherapy targets fast-growing or fast-dividing
cells, it is more likely to harm similar cells in the body. These include the cells in the hair follicles, which are why cancer treatment is often associated with hair loss, although hair does regrow once treatment has ended. Other fast-dividing cells can be found in the stomach and bowel lining, which leads to nausea and diarrhea. There are, however, drugs which help control this, and timing meals to avoid having a full stomach when the drugs take effect can also help in some cases. Other types of normal cell that can suffer are the blood cells. Red cells are important to carry oxygen to keep other cells alive. Other blood cells help stave off infection. As a result, chemotherapy patients may be more prone to infections, and find them harder to fight off.

Convention chemotherapeutic or radiotherapeutic regimens often fail to cure patients because they do not kill all malignant cells. One reason is that the dose needed to eradicate cancer is so large that patient’s own tissues would be severely damaged. Active specific immunotherapy with antigenic tumor cells, (rendered non tumorigenic by e.g. irradiation) or extracted tumor associated antigens is another approach for improved systemic cancer therapy

1.1.3. IMMUNOTHERAPY OF CANCER

The concept of the immune system being involved in the development of cancer and of manipulating it as a part of cancer therapy dates back almost 100 years. For immune surveillance against cancer to be a plausible theory, cancer cell must express antigens recognizable as foreign and accessible to the immune system, which must in turn be able to mount a response against cells bearing such antigens. Immunotherapy is treatment by immunological means. The aim of treatment in cancer is to eliminate the tumor without harming the host.
Tumor immunity can be enhanced by providing co-stimulatory signal necessary for activation of Cytotoxic-T Lymphocytes precursors (CTL-Ps). When mouse CTL-Ps are incubated with melanoma cells in vitro, they do not proliferate and differentiate into effector CTLs, but when melanoma cells are transfected with gene encoding the B7 ligand, then the CTL-Ps differentiate into effector CTLs (6). The identification of novel B7-family members, the modulation of CD40 to reverse tolerance to tumor-associated antigens and the use of CX40 to enhance antitumor response of CD4+ T cells have all contributed to the development of more powerful immunomodulatory cancer therapies (55). In human chronic myeloid leukemia (CML) model, tumor cells incubated in Granulocyte Macrophages-Colony Stimulating factors (GM-CSF), IL-4 and TNF differentiated into mature dendritic Cells capable of stimulating an autologous anti-CML CTL response (30). Some malignant cells in patients with acute myelogenous leukemia, upon cytokine administration (i.e. GM-CSF or IL-4), undergo differentiation into APCs that contain high levels of co-stimulatory and MHC molecules. These cells are ideal Antigen Presenting Cells as presumably the entire repertoire of tumor antigens is presented on them (23, 15).

The dendritic cells of mouse when cultured in GM-CSF and incubated with tumor fragments have been shown to activate both T-helper (T_h) cells and CTLs specific for the tumor antigens. When these mice were subsequently challenged with live tumor cells, they displayed tumor immunity. In one approach, tumor cells were transfected with GM-CSF gene (26). These engineered tumor cells, when reinfused back into the patient, secrete GM-CSF, enhancing the differentiation and activation of host antigen-presenting cells, especially dendritic cells. As these dendritic cells accumulate around the tumor cells, the GM-CSF secreted by the tumor cells will enhance the presentation of tumor antigens to T_h and CTLs cells by the dendritic cells. More recently, it has been found that dendritic cells (DC) phagocytose apoptotic influenza-infected monocytes and cross-present influenza antigen to CD8+ T cells, generating a specific CTL response (60).
Several adjuvants, such as attenuated strain of Mycobacterium bovis called bacillus Calmette-Guerin (BCG) and Corynebacterium parvuum, are used to boost tumor immunity. These adjuvants activate macrophages increasing their expression of various cytokines, class II MHC molecules, and the B7 co-stimulatory molecule. Thus, the macrophages activates $T_H$ cells, resulting in generalized increases in both humoral and cell-mediated responses.

A variety of experimental and clinical approaches have been developed to use recombinant cytokines, either singly or in combination, to augment the immune response against cancer. Various cytokines used in cancer immunotherapy are interferon $\alpha$, $\beta$ and $\gamma$, IL-1, IL-2, IL-4, IL-5, IL-12, and IL-15, GM-CSF and TNF (82,63,65,67). Purified recombinant preparations of the interferon, IFN-$\alpha$, IFN-$\beta$ and IFN-$\gamma$ are available, each of which has shown some promise in the treatment of human cancer. To date, most of the clinical trials have involved several mechanisms. All three types of interferon have been shown to increase class I MHC expression on tumor cells; IFN-$\gamma$ have also been shown to increase class II MHC expression on macrophages. Given the evidence for decreased levels of class I MHC molecules on malignant tumors, the interferons may act by restoring MHC expression, thereby increasing CTL activity against tumors. Interferons have been shown to inhibit cell division of both normal and malignantly transformed cells in vitro. IFN-$\gamma$ increases the activity of $T_C$ cells, macrophages, and NK cells, all of which play a role in the immune response to tumor (6).

Tumor necrosis factors, TNF-$\alpha$ and TNF-$\beta$, have been shown to exhibit direct antitumor activity, killing some tumor cells and reducing the rate of proliferation of others while sparing normal cells. In the presence of TNF-$\alpha$ or TNF-$\beta$ a tumor undergoes visible hemorrhagic necrosis and tumor regression. TNF-$\alpha$ has also been shown to inhibit tumor-induced vascularization (angiogenesis) by damaging the vascular endothelial cells in the vicinity of tumor, thereby decreasing the flow of blood and oxygen that is necessary for progressive tumor
growth. Injection of TNF-α directly into the tumor has led to complete tumor regression in some patients, but not in others. TNF-α therapy has several limitations: the short half-life of TNF-α necessitates frequent injections; and its adverse side effects include fever, chills, blood-pressure changes, and decreased counts of white blood cells. (65).

Monoclonal antibodies have been used in various ways as experimental immunotherapeutic agents for cancer (42). Anti-idiotype monoclonal antibodies have been used with some success in treating human B-cell lymphomas and T-cell leukemias. Monoclonal antibodies also have been used to prepare tumor-specific immunotoxin (82). These agents consist of the inhibitor chain of a toxin (e.g. diphtheria toxin) linked to an antibody against a tumor-specific or tumor-associated antigen. In vitro studies have demonstrated that those "magic bullets" can kill tumor cells without harming normal cells.

Another cancer immunotherapy involves using monoclonal antibodies to bridge activated T cells directly to a tumor. In this approach two different monoclonal antibodies are produced: one specific for a tumor-cell membrane molecule and other specific for the CD3 membrane molecule of the TCR complex. A hybrid monoclonal antibody, or heteroconjugate, is then prepared with specificity for the tumor antigen and for CD3. In vitro experiments with these hetero-conjugates have revealed that they are able to cross-link and activate T cells directly on the surface of the tumor cell. A variety of tumors express significantly increased levels of growth-factor receptors suggests that treatment with monoclonal antibodies against receptors might inhibit tumor-cell activity. Monoclonal antibodies to the EGF receptors; to the p97 (transferrin) receptor, and to the IL-2 receptor have been produced. In the present study specific active immune response was induced against tumor antigen in mice bearing tumor. The tumor was initiated by chronic exposure of chemical carcinogen to experimental animals.

1.2 CHEMICAL CARCINOGENS
Substances capable of producing neoplasms are classified as carcinogens. Carcinogens are reactive intermediates, as electrophilic reactants or radical cations, that can interact with cellular macromolecules (130). They include a highly diverse collection of chemical substances, both organic and inorganic chemicals, solid-state materials, hormones, and immunosuppressants (83). Chemical carcinogens can be classified into two major categories - genotoxic and epigenetic.

Genotoxic carcinogens are identified by the biochemical demonstration of DNA damage or by the demonstration of genotoxic effects in short-term tests. Most likely, alteration in DNA is the key event in the initiation of carcinogenicity by these compounds. Genotoxic carcinogens are occasionally effective after a single exposure and frequently carcinogenic at subtoxic doses. They act in a cumulative manner together with other DNA-reactive carcinogens having the same organotropism. They usually produce neoplasms in more than one target organ and have a short latent period. Examples of genotoxic carcinogens are ethyleneimine, dimethylnitrosamine, and diethylnitrosamine, vinyl chloride and nickel (129).

The second broad category, designated as epigenetic agents, comprises those chemicals for which no evidence exists of direct interaction with genetic material, but which produce another biological effect that could be the basis for their individual carcinogenicity. Possible mechanisms for epigenetic effects include chronic tissue injury, hormonal imbalance, immunologic effects, or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens (129). This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants, and promoters. Examples of epigenetic carcinogens include nitrolotriacetic acid, asbestos, phorbol esters, Estradiol, Azothioprine.
The production of cancer in humans and animals by chemical carcinogens is thought to be the end result of a complex series of individual reactions that are subject to and controlled by a number of modifying factors. The reactions can be grouped into two sequences. In the first sequence, the normal cell is converted to a neoplastic cell, and in the second sequence, the neoplastic cell develops into an overt neoplasm. In the neoplastic conversion different enzyme systems can function in the detoxification and elimination of xenobiotics (128). Many carcinogens, however, undergo enzymatic activation to a reactive ultimate carcinogen. A small number of carcinogens, mostly industrial intermediates and chemotherapeutic drugs, are reactive in their parent form and therefore do not require activation. Carcinogens that form reaction reactive species undergo covalent reactions with a variety of cellular macromolecules, including DNA (48). If the cell replicates while DNA damage is persistent, permanent alterations in the genome can be produced in several possible ways: the mispairing of bases leading to point mutations; errors in replication yielding frame-shift mutations; transpositions resulting in codon rearrangement; and combinations of these alterations in sequential steps. Codon rearrangement may involve sequences known as oncogenes, which are emerging as critical gene sequences for transformation (127). In the case of interactions with the mitotic apparatus, chromosomal mutations and aneuploidy could result. All these alterations generate a permanently abnormal cell with an altered genotype and distinct phenotypes.

Thus, the abnormal cell formed may be held in check by tissue homeostatic factors or, if the conditions of exposure or abnormalities generated in the cells permit, they may undergo limited proliferation to form "preneoplastic" lesions. Now, cells with the requisite abnormalities may have the capacity to proliferate beyond tissue constraints to form neoplasms. Neoplasms can undergo qualitative changes in their phenotypic properties, possibly including transition from benign to malignant behavior (41). This change probably reflects the selection during growth of a population with a genotype coding that produces advantageous phenotypic...
properties. New genotypes could arise in neoplasms through errors in DNA replication (133) or alterations in chromosome constitution.

1.2.1 CARCINOGENICITY OF DIETHYLNITROSAMINE

Diethylnitrosamine (DEN) is a synthetic derivative of nitrosamine. It is an established and potent hepatocarcinogen. The carcinogenic activity of this compound was shown by Schmahl and Preussman (109) in 1960. The LD50 for DEN is 216 mg/kg when administered by intraperitoneal route injection in rats. The lowest published toxic dose is 100 mg/kg body weight when administered intraperitoneally (25). The acute toxic effects from exposure to DEN are similar to those produced by dimethylnitrosamine with serious destruction of liver tissue as the most important result (77). There is no correlation between acute toxic effects and carcinogenic potential for nitrosamines. This is demonstrated by the fact that even though DEN has only about one-sixth the acute toxicity of dimethylnitrosamine (27), if administered continuously to rats, it is probably a more active liver carcinogen (28).

DEN has been shown to be carcinogenic to the mouse, the rat, the hamster, the guinea-pig, the rabbit, the dog, the pig, the monkey, and to aquarium fish. The agent induces tumors primarily in the nasal cavity, trachea, esophagus, and liver. It causes cancer after different modes of exposure, which include ingestion, inhalation, and skin painting. It is carcinogenic in single doses and following prenatal exposure. In lifetime feeding studies with rats in which daily doses between 1 and 10 mg/kg body weight were administered, tumor yields approaching 100% have been obtained (56).

In a dose-response study conducted using rats DEN was administered in drinking water and the daily exposure was between 0.075 and 14.2 mg/kg body weight in 9 groups of animals. The total dose, until death occurred, was between 64 and 965 mg/kg body weight. The tumor
induction time was between 68 and 840 days. All daily doses higher than 0.15 mg/kg body weight gave a tumor incidence of 100%. When a dose of 0.15 mg/kg body weight per day was administered, a tumor yield of 90% was obtained. At 0.075 mg/kg body weight per day, 20 rats survived for more than 600 days and 11 of the 20 animals had tumors of the liver, esophagus, or the nasal cavity. All 4 of the animals that lived longer than 940 days at this dose level had tumors (56).

1.2.2 STRUCTURE AND PHYSICAL PROPERTIES OF DEN

\[\begin{align*}
\text{H}_2 & \\
\text{H}_3\text{C} - & \text{C} \\
\text{N} - & \text{N}=\text{O} \\
\text{H}_3\text{C} - & \text{C} \\
\text{H}_2
\end{align*}\]

mol wt: 102.1

physical appearance: a yellow, volatile liquid

boiling point: 177°C (760 mm Hg) 64-65°C (17 mm Hg)

density: 0.9422 (20/4°C)

refractive index: 1.4386 (20°C)

solubility: about 10% in water; soluble in organic solvents and in lipids.

1.2.3 METABOLIC ACTIVATION OF DEN

Diethylnitrosamine must be metabolically activated to reactive intermediates to interact with cellular macromolecules and initiate carcinogenesis. The metabolic activity occurs in the microsomes of liver and in presence of \(O_2\) and NADPH forms the intermediate monodiethylnitrosamine upon oxidative deethylation (29). Diethylnitrosamine-ethylase is the probable activating enzyme, with acetaldehyde as the metabolic product. Mirzahi and Emmelot
(89,88) were the first to study the properties of DEN-ethylase. The metabolic activation of DEN follows the pattern of DMN—monodealkylation followed by the liberation of a reactive ethonium ion from the remainder of the molecule as demonstrated by Dutton and Heath (29). The α-hydroxylation hypothesis is currently accepted mechanism of metabolic activation of DMN.

Mechanism of metabolic activation of diethylnitrosamine

Hydroxylation of DEN (29), at the α-carbon is believed to be the critical, rate-limiting step. The putative α-hydroxylated diethylnitrosamine (ii) is extremely unstable and yields, upon hydrolysis, acetaldehyde and monoethylnitrosamine (iii). The overall reaction is N-deethylation. Monoethylnitrosamine (iii) is also highly unstable and readily undergoes non-enzymatic, spontaneous rearrangement or breakdown to a “ethylating intermediate”, which, is assumed to be carbonium ion (vi), although the possibility that the ethylating intermediate may be monoethylnitrosamine (iii) itself, or its tautomeric form, ethyldiazenium hydroxide (iv) or diazoalkene (vii) has been suggested at various times.
Thus, the liberation of the reactive ethonium ion which, is an electrophile ethylates with the nucleophilic centers in vivo of DNA, RNA and protein. The ethylation of rat liver DNA by DEN has been studied by Scherer et al (107). The potential adduct found include $O^2$- and $O^4$- ethylthymidine, and $O^6$- methylguanine.

1.3 TUMOR ASSOCIATED ANTIGENS (TAA)

Antigens of tumors induced by chemical carcinogens exhibit little or no immunologic cross-reactivity; each tumor exhibits a unique antigenic specificity. Thus, cells of a given tumor, arising from a single transformed cell, all share common antigens, but different tumors, even if induced by the same carcinogen, are antigenically distinct from one another. This absence of cross reactivity is probably due to the random mutations induced by the chemical or physical carcinogens, leading to a large array of different antigens. (10)

Tumor-specific transplantation antigens (TSTAs) or simply tumor-specific antigens (TSAs), are antigens found on the surface of malignant cells and consist of structures that are unique to the cancerous cells and are not present on their normal counterparts. The existence of these antigens comes from the study of transplanted tumors in inbred animals. Some of these tumors express antigens that elicit T cell-mediated immune rejection responses in syngeneic hosts. These antigens can be specific for individual tumors or common to a class of tumors (115).

Antigens on tumor cells are usually also found on normal cells. The expression of such molecule on tumor cells, however differs from the expression on normal cells. For instance, they are present on high levels on tumor cells and in trace amount on normal cells, they are usually distributed over the cell membrane or they are expressed in an appropriate phase of
ontogenesis. Little evidence is available on the existence of real tumor-specific antigens in man. Therefore, antigens on tumor cells are usually tumor-associated antigens (TAA).

TAA represent a heterogeneous group of macromolecular structures (glycoproteins, glycolipids) in or on tumor cells (69) and can serve as effective targets for active immunotherapy against tumor. In this context, preparations containing extracted TAA of tumor cells (TAA extract) and purified TAA have been explored for active immunization. Encouraging results have been found.

Extraction of viable tumor cells with low concentrations of butanol has become increasingly popular for obtaining TAA. With this procedure mainly peripheral membrane components are released, thus avoiding extraction of cytoplasmic or integral membrane components (68). Butanol extraction does not exert cytolytic effects as viability and proliferation capacity of the tumor cells is preserved.

1.4 TAA BASED IMMUNOTHERAPY

One approach in immunotherapy is specific active immunotherapy with antigenic tumor cells which have been rendered non-tumorigenic or extracted tumor-associated antigens (TAA). The objective being to stimulate the host immune response against the tumor (80,19). Antigens on tumor cells are usually also found on normal cells. The expression of such molecules on tumor cells, however, differs from the expression on normal cells, they are usually distributed over the cell membrane, or they are express at an inappropriate phase of oncogenesis, as in the case of fetal antigens on tumor cells in adults.

One of the main problems in specific active immunotherapy is the generally weak immunogenicity of TAA (67). Therefore, a major challenge in tumor immunology is to develop
methods that augment the immune response to TAA. Different methods have been explored. Tumor cells have been modified to enhance their immunogenicity. This has been accomplished in various ways, for example, by enzymatic unmasking of TAA with enzymes such as neuraminidase (111), by additional immunogenic cell surface determinants (50), or by rigidification of cell membranes with lipids (113). Immunity against these more immunogenic tumor cells usually cross-reacts with the original tumor cells. Thus, this approach may lead to success. A second approach is to employ immunostimulants as immunological adjuvants. \textit{Bacillus Calmette-Guerin} (BCG) and \textit{Corynebacterium parvum} mixed with irradiated tumor cells have been especially studied in the past (53). More recently better defined and probably less toxic immunostimulants, such as cytokines and analogs of bacterial products, are under investigation (75,76,81). A somewhat different approach involves the reduction of suppressor T cells. This kind of treatment is based on observations that tumor-bearing animals may have T cells that can specifically suppress the immunological response to TAA (95). Most of the attention is focus upon cyclophosphamide to achieve this goal.

Instead of whole tumor cells, preparation containing extracted TAA of tumor cells (TAA extract) have recently been explored for active immunization (40,62). Immunization with purified TAA may be more effective than whole tumor cells. In the latter case, the relevant TAA may be present at a low density and consequently swamped by other determinants. The recently isolation and characterization of certain TAA enable the study of immune reactions against highly purified TAA with encouraging results in cancer patients (99,51).

\section*{1.5 USE OF LIPOSOMES IN TUMOR IMMUNOTHERAPY}

The TAA responsible for mediating tumor rejection are integral membrane molecules or molecule associated with the tumor cell surface. Their immunogenicity is closely related to their presentation form. Liposomes have been shown to potentiate the immune response of a
variety of antigens. Liposomes are artificially prepared spheres, consisting of concentric phospholipids bilayers separated by aqueous compartments. Liposomes have been first described by Alec D. Bangham (13). They are formed when phospholipids are confronted with water. The phospholipid molecules prefer to find a conformation in which their hydrophobic fatty acid chains are prevented from contact with water. For that reason, phospholipid are formed in which the relatively hydrophilic head groups are made up of both of the outer parts of each bilayer, whereas the hydrophobic fatty acids groups are located directly opposite each other as the inner part of the bilayer.

Part of the aqueous solution, together with hydrophilic molecules dissolved in it will be encapsulated during the formation of the liposomes, whereas lipophilic bilayers molecules may be associated with the phospholipid bilayers. Amphipatic molecules, as the phospholipids themselves, attempt to find a conformation, with their hydrophilic parts extended in the aqueous compartments and their hydrophobic parts inserted in the bilayers. Liposomes may differ in their dimensions, composition (different phospholipids and cholesterol contents), charge (resulting from the charges of the composing phospholipids), and structure (Uni- or multilamellar liposomes consisting of only one or more phospholipid bilayer surrounding one aqueous compartment).

The in vivo distribution of liposomes are biodegradable and may be composed of nontoxic and immunologically inert phospholipids, they have been suggested as promising carrier for haptens and antigens. Immunopotentiation has been established both for antigens exposed on liposomal outer surfaces and for antigens encapsulated within the liposomes. Interest centered on the possible applicability of liposomes as an adjuvant for the preparation of vaccines and for the preparation of tumor antigens to the immune system (122)
The immunopotentiating ability of liposomes has been first shown by Allison and Gregoriades in 1974. They have reported the adjuvant effect of liposomes in eliciting antitoxin diphtheria toxoid immune response (5). Subsequently various workers confirmed the immunoadjuvanicity of liposomes in case of proteins (123), peptides (73), lipids (8) and carbohydrates (108).

Thus, liposomes may be an effective tool to obtain enhanced immune reactions against TAA by acting as an adjuvant, because effective immunotherapy employing TAA depends upon reconstitution into an environment that enhances immunogenicity. Direct interaction between liposome-associated antigens and lymphocytes is well documented for the in vivo stimulation of T-cells (126). A likely prerequisite for direct stimulation of lymphocytes by liposome-associated antigens is the presentation of the antigens on the outermost layer of liposomes. However, the antigen might also be incorporated into the interior of the liposomes and consequently, not directly available for interaction with immunocompetent cells. Thus the immune response evoked by liposomal antigens can also be the result of their targeting to the antigen-presenting cells.

Various technical problems have impeded the development of liposomes as viable clinical tools for the delivery of cytotoxic antineoplastic agents to tumor e.g., the poor entrapment efficiency of drugs, increase uptake by liver and spleen macrophages and hepatocytes and the disintegration into blood. However, encouraging progress have been made in overcoming some of these problems. Recently, a method to produce liposomes capable of achieving high entrapment efficiency was developed and has been successfully used for entrapment of toxin, radiomodulators and radiosensitizer (110). The method is highly reproducible and the biological activity of the entrapped material was found to remain unaltered. In many experimental situations the immunogenicity of the antigen-containing liposomes was still relatively low. Therefore, immunostimulants were applied as adjuvants to
further potentiate the immune response. Lipoidal adjuvants that activate macrophages have been studied extensively. Lipid A and lipophilic derivatives of MDP, in particular have shown to be effective (18). Some new lipophilic MDP derivatives and the sodium phthalyated derivative of lipopolysaccharide (SPLPS) have been found potent adjuvants in stimulation of immune responses (2,3,105,112,121). There is no doubt that antibody response can be induced by TAA containing liposomes. However, the potential of the antibodies to mediate tumor regression is still a matter of controversy. On the other hand, antibodies may kill tumor cells in vitro by complement dependent lysis or by antibody mediated cell-dependent mechanisms. Clinical responses in some patients treated with monoclonal antibodies have been observed (85,58). On the other hand, the presence of antibodies on tumor cells is regarded as unfavorable as they can block the immune response in vivo or can form antigen-antibody complexes that can induce suppressor cells (94). Generally, it is believe that in most case cell-mediated immune response rather than humoral response is of prime importance in tumor rejection. Therefore, in order to induce both types of immune responses in host against TAA, incorporation of macrophage activators such as MDP, INF-r, and IL-2 or B-cell mitogen such as SPLPS in the liposomal TAA formulation seems realistic.

Little information is available on the mechanism behind the potentiation of antitumor immune responses after incorporation of TAA into liposomes. In general, antigen presenting cells (APCs) present TAA in association with the proper MHC molecules to helper cells. This activates T-cells to the production of nonspecific T cell factors, the lymphokines. These factors participate in the induction of cytotoxic macrophages, natural killer (NK) cells, cytotoxic T cells, and Ig synthesis by B cells. It is not known to what extend these effectors mechanisms are stimulated by liposomal TAA and whether these mechanisms can be manipulated by the liposomal characteristics. The immunopotentiation of TAA by liposomes might be explained by a better targeting of TAA to antigen presenting cells and consequently, improved stimulation of one or more tumoricidal effector mechanisms.
Rodent and human macrophages activated by various immunomodulators acquire the ability to destroy neoplastic cells in vivo while leaving non-neoplastic cells unharmed. Macrophages are also an important component of both the afferent and efferent arms of the immune system and can also be important in the defense against neoplasm (39). Liposomes provide a particularly convenient non-toxic carrier for the delivery of biologically active materials to mononuclear phagocytes in vivo (40). Following i.v. administration, liposomes are taken up by the reticuloendothelial (RE) cells in the liver and spleen by circulating monocytes (96). By exploiting this localization pattern, it is possible to target liposome-encapsulated materials to macrophages in vivo. Preferential delivery of the liposome-encapsulated substance can be achieved in vivo because mainly phagocytic cells are exposed to the liposome-entrapped antigens. Once phagocytosed, biological active materials are release into the cytoplasm of the phagocyte, thereby avoiding the problem of dilution, serum protein binding, and rapid clearance, and minimizing the elicitation of undesirable side effects. This natural localization pattern also allows efficient targeting of liposomes and their contents to various macrophage components in the body. Therefore, the systemic activation of macrophages by liposome-encapsulated TAA and supplemented by immunostimulator can provide a successful approach to the eradication of established cancer metastases.

The present study uses tumor bearing animal model for TAA vaccination in an attempt to devise an effective formulation using liposomes that would elicit both humoral and cellular responses against tumor. The following objectives were conceived for the investigation embedded in this thesis.

1. Induction of Tumor in mice by diethylnitrosamine (DEN)
2. Isolation of Tumor-Associated Antigens
3. Encapsulation of TAA into Liposomes
4. Stimulation of Immune Reaction against Liposomal-TAA
5. Study The Effect Of Immune Responses and Assessment Of Tumor Regression.