Cytotoxic Agents with Immunosuppressive Activity

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A whole variety of chemical and biological materials have immunosuppressive effects in animals. Although anti-inflammatory agents, especially the corticosteroids, are inhibitors of the immune response, most are general cytotoxic agents.

The cytotoxic agents were first developed as antitumour agents. It is now well known that they are not particularly selective for cancer cells, but are selectively toxic for cells that are in cycle. Some are even more specific, acting only on cells in a particular phase of the cell cycle. When selective antitumour effects are obtained, this is not through a different mechanism of action, but is a result of other properties of the agent that ensure that a greater reaction takes place in the cancer cell than in sensitive cells of the host. This may be achieved by selective concentration or by selective activation of the agent in the cancer cell. Alternatively the compound may be more readily detoxified by normal cells or these may have a greater ability to repair any damage caused. As a result of their general cytotoxicity these antitumour agents have a number of side effects, most of which are undesirable, but which may sometimes be exploited, such as in their use as immunosuppressive agents. When these agents are used for their immunosuppressive properties their selectivity is once more determined by the properties of the whole molecule, which regulate the amount of agent reacting with the target sites of the immunocyte compared with the amount that reacts with sensitive host cells.

Cytotoxic agents

Scheme 1 summarizes the cytotoxic agents that have been used to suppress various types of immune response, either experimentally or clinically, and the cellular sites at which they act. The scheme shows biosynthesis of ribonucleotides from simple precursors and their reduction to form deoxyribonucleotides, which are polymerized to DNA. Antimetabolites generally act by inhibiting purine and pyrimidine biosynthesis de novo at early stages. Some can also specifically inhibit the formation of dTMP from dUMP. Cytosine arabinoside also acts at a later stage by inhibiting DNA polymerase, and some of the effects of the base analogues are due to their incorporation into nucleic acids. Many different chemical classes cause cytotoxicity by reacting covalently with DNA. Usually the compounds are electrophilic reactants and combine covalently with nucleophilic sites in DNA. A number of antibiotics that bind strongly to DNA are also immunosuppressive. Puromycin acts at the level of the ribosomes, and Vinca alkaloids and related spindle poisons directly on microtubular proteins. Asparaginase exerts all its effects extracellularly, and is specifically cytotoxic to these cells that are deficient in the asparagine synthetase enzyme and that therefore require asparagine as an essential amino acid (Broome, 1970).

Because of their general action against dividing cells, the cytotoxic agents cause a non-specific immunodepression and act particularly on the small sensitized lymphocyte rather than on the long-lived lymphocyte (Gerebtzoff et al., 1972).

Alkylating agents

Alkylating agents act on a variety of cell nucleophiles, including amino acids, nucleosides, nucleotides, proteins and nucleic acids. Several different types have been studied for their immunosuppressive activity, including nitrogen mustards, ethylene-imines (aziridines) and sulphonoxalkanes (Gerebtzoff et al., 1972; Camiener & Wechter, 1972). Besides being alkylating agents reactive under physiological conditions, they are all at least bifunctional, i.e. have at least two alkylating groups in each molecule. Alkylation takes place by a second-order nucleophilic substitution (S_N2) in most cases. The aromatic nitrogen mustards show different kinetics and possibly act...
Scheme 1. Cytotoxic agents that have been used to suppress various types of immune response, and the cellular sites at which they act.
through a carbonium ion formed by unimolecular loss of halogen ions, thus resembling an $SN_1$ nucleophilic substitution.

Although many cell molecules are alkylated in vivo, most of the evidence favours DNA as the essential target site. A cross-linking reaction between two bases, which can only occur with agents that are at least bifunctional, is suggested as the most cytotoxic lesion (Ross, 1962; Brookes & Lawley, 1964).

Cyclophosphamide (I) is the most widely used of alkylating agents, and although its mechanism of action is the same it is the most selective in its immunosuppressive properties. It is only a weakly active alkylating agent because of the electron-withdrawing properties of the oxazaphosphorine ring. It is believed to be metabolized in vivo, predominantly in the liver, to the 4-hydroxy derivative (II), which may then be enzymically converted into a ring-opened propionic acid derivative or the 4-oxo compound. None of these metabolites is a cytotoxic agent. However, the 4-hydroxy derivative may also break down spontaneously by $\beta$-elimination to acrolein (acrylic aldehyde) and phosphoramid mustard (III), both of which are highly cytotoxic (Connors et al., 1973). Selectivity occurs probably because there is a greater chemical breakdown to the cytotoxic products in the lymphocyte than in other sensitive host cells.

Other alkylating agents

Several other classes of agent are known that also react with DNA. They are distinguishable from the alkylating agents because some are only monofunctional or have a different mechanism of action, and they differ in their antitumour properties. A platinum complex, cis-dichlorodiammineplatinum (IV), has been shown to suppress a variety of immune responses (Berenbaum, 1971; Khan & Hill, 1971), and a number of new derivatives have since been synthesized. Bis(chloroethyl)nitrosourea (V) and related compounds can decompose to alkylating agents and also to products that interfere with protein synthesis (Carter et al., 1972), and both mechanisms may cause cytotoxicity. Carcinogenic polycyclic aromatic compounds such as 7,12-dimethylbenz[a]anthracene suppress delayed hypersensitivity and humoral responses in laboratory animals (Stjernsward, 1969). It is likely that all carcinogens of this type are converted into alkylating agents in vivo. Natulan (VI) is known to decompose in vitro in the presence of $O_2$ into a number of products that react with DNA, and this is the basis of its mechanism of action in vivo.

Antifolates

The most widely used antifolate is methotrexate (VII), which is essentially an irreversible inhibitor of the folate reductase enzymes (Balis, 1968). A number of important biosynthetic pathways are inhibited and there are effects on early stages of purine synthesis. The most critical effect, which is probably mainly responsible for cytotoxicity, is inhibition of the formation of dTMP from dUMP, a result of a deficiency of $N^2N^{10}$-methylene tetrahydrofolate. Many compounds are known to have antifolate activity; most are structural analogues of folic acid, but none has replaced methotrexate in the clinic.

Antipyrimidines

5-Fluorouracil (VIII), after conversion into the nucleotide and deoxynucleotide, inhibits thymidylate synthetase, preventing the synthesis of dTMP and consequently inhibiting DNA synthesis. The corresponding bromo- and iodo-riboside act similarly to 5-fluorouracil, and are also incorporated as base analogues into DNA, replacing the thymidine by as much as 50% of the total thymidine content. The primary action of the bromo- and the iodo-riboside may be a result of their extensive incorporation into DNA as the deoxyribotides.
Cytosine arabinoside (IX) is an effective immunosuppressant when given in multiple doses. Like most antimetabolites, it acts on a number of different stages of nucleic acid synthesis, but it probably causes cytotoxicity by inhibiting a DNA polymerase.

**Antipurines**

6-Mercaptopurine (X), one of the first purine analogues to be synthesized, acts after conversion into its ribonucleotide, by preventing the anabolism of IMP. The lack of AMP and GMP prevents the synthesis of both DNA and RNA. 6-Mercaptopurine may also be incorporated into nucleic acid, as 6-thioguanine deoxynucleotide, which is an immunosuppressive agent in its own right. An imidazole derivative of 6-mercaptopurine, azathioprine (XI), is one of the most widely used immunosuppressive drugs. *In vivo* it slowly liberates 6-mercaptopurine, but it does have advantages over this compound, and the selectivity of azathioprine may, in fact, be due to differential rates of formation of 6-mercaptopurine in different cells. Cleavage of the imidazole group may not be a prerequisite for activity, since a number of 9-substituted derivatives are active in their own right; although not dealkylated *in vivo*, they are good immunosuppressive agents (Gerebtzoff *et al.*, 1972).

**Other agents**

Azaserine (XII) is a glutamine antagonist that has been used clinically and acts principally by preventing the formation of formylglycinamidine ribonucleotide, an early stage of purine biosynthesis. A related compound, 6-diazo-5-oxo-L-norleucine, is more potent, binding 40-fold more strongly to the same enzyme. Hydroxyurea has the biological properties of an antimetabolite and causes a specific inhibition of DNA synthesis. This is a result of an interaction between hydroxyurea and the protein units that aggregate in the presence of metal ions to form the enzyme ribonucleotide diphosphate reductase (Reichard, 1967).

**Antibiotics**

Most antitumour antibiotics, such as actinomycin D, daunomycin and the related adriamycin, bind strongly to DNA. All have a planar polycyclic nucleus as a part of their structure, together with a polypeptide or carbohydrate chain or other bulky group. The polycyclic nucleus has the appropriate groups so placed that they can hydrogen-bond very strongly to purines and pyrimidines. All these agents intercalate DNA and sometimes RNA, a molecule of the antibiotic fitting between two stacked base-pairs, causing a distortion of the double helix and consequently interfering with the function of DNA polymerases and RNA polymerases. Puromycin, which has also been used as an immunosuppressant, is an inhibitor of protein synthesis, inducing the release of incomplete proteins from the ribosomes.

**Spindle poisons**

A number of natural products, such as colchicine and podophyllotoxin, arrest cells in metaphase by interfering with the formation of the mitotic spindle apparatus. The *Vinea* alkaloids are a member of this class and they have a variety of immunosuppressive effects. Their mechanism of action is not fully known, but they appear to form a complex with microtubular protein, preventing it from condensing to form various cell components. A specific effect in metaphase is obtained probably because the cells have an increased permeability to the alkaloids just before mitosis. At this time the microtubular protein is being synthesized for the formation of the spindle apparatus.

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The Development and Production of Anti-Lymphocytic Globulin for Clinical Trial

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Several years ago the Wellcome Foundation undertook to supply, to the Medical Research Council Working Party on anti-lymphocytic serum, the serum necessary for the conduct of a clinical trial on the effect of anti-lymphocytic serum on transplant survival. At that time it was envisaged that it was only necessary to inject a few horses with human spleen cells, wait a few weeks, bleed the animals and isolate the immunoglobulins from the serum. The immunoglobulins were expected to be available in some 6 months after the first meeting of the Working Party. In the event, it has taken over 6 years to reach the clinical-trial starting point, and it is with some of the reasons for the time-lag that this paper deals.

The advantages of anti-lymphocytic serum/globulin over other forms of immunosuppression have been presented by Medawar (1969). These include the fact that, alone among immunosuppressives, anti-lymphocytic serum abrogates the normal lymphocyte transfer reaction, it does not rely on a proliferative phase of the response for its action and it is much less sensitive than other agents to the antigenic differences between donor and recipient. Such considerations and the undesirable sequelae of continual large doses of steroids led several groups to use anti-lymphocytic serum clinically despite the known hazards, such as anaphylaxis and serum sickness, of the use of heterologous serum. The pioneering experimental work of Woodruff & Anderson (1963, 1964), who demonstrated in rats that skin-graft survival could be prolonged by the use of anti-lymphocytic serum, laid the way for a mass of experimental work confirming the effectiveness of this antiserum, especially in rodents (for references see James, 1969). This encouraged several groups to proceed to make anti-(human lymphocyte) serum.

A major part of the work reported at that time came from Starzl and his colleagues, who reported beneficial results of using intramuscular injections of anti-(human lymphocyte) globulin preparations. The preparations (Starzl et al., 1967) were made by using a mixture of thymus, lymph-node and spleen cells as antigen and horses as the source of the serum. The purified globulins produced considerable pain and swelling at the injection sites, and Long et al. (1969) have pointed out that stimulation of the adrenal cortex to release steroids could have accounted for the apparent immunosuppressive effect in Starzl's patients. Nevertheless Starzl's series of patients represented at the time the most completely documented and successful series yet reported, and it seemed the right course to attempt to follow his lead. Spleen cells were the only material available to us as the antigen and were used in our first attempts to prepare anti-lymphocytic globulin for the Medical Research Council Working Party.

Spleen cells were obtained at operation or at post-mortem. Those obtained at operation, and processed immediately at the hospital and sent to us as a cell dispersion,