the mode of action of immunosuppressive agents

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THE MODE OF ACTION OF IMMUNOSUPPRESSIVE AGENTS

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Editor's preface

The aim of the publication of this series of monographs, known under the collective title of *'Frontiers of Biology'*, is to present coherent and up-to-date views of the fundamental concepts which dominate modern biology.

Biology in its widest sense has made very great advances during the past decade, and the rate of progress has been steadily accelerating. Undoubtedly important factors in this acceleration have been the effective use by biologists of new techniques, including electron microscopy, isotopic labels, and a great variety of physical and chemical techniques, especially those with varying degrees of automation. In addition, scientists with partly physical or chemical backgrounds have become interested in the great variety of problems presented by living organisms. Most significant, however, increasing interest in and understanding of the biology of the cell, especially in regard to the molecular events involved in genetic phenomena and in metabolism and its control, have led to the recognition of patterns common to all forms of life from the bacteria to man. These factors and unifying concepts have led to a situation in which the sharp boundaries between the various classical biological disciplines are rapidly disappearing.

Thus, while scientists are becoming increasingly specialized in their techniques, to an increasing extent they need an intellectual and conceptual approach on a wide and non-specialized basis. It is with these considerations and needs in mind that this series of monographs, *'Frontiers of Biology'*, has been conceived.

The advances in various areas of biology, including microbiology, biochemistry, genetics, cytology, and cell structure and function in general will be presented by authors who have themselves contributed significantly to these developments. They will have, in this series, the opportunity of bringing

together, from diverse sources, theories and experimental data, and of integrating these into a more general conceptual framework. It is unavoidable, and probably even desirable, that the special bias of the individual authors will become evident in their contributions. Scope will also be given for presentation of new and challenging ideas and hypotheses for which complete evidence is at present lacking. However, the main emphasis will be on fairly complete and objective presentation of the more important and more rapidly advancing aspects of biology. The level will be advanced, directed primarily to the needs of the graduate student and research worker.

Most monographs in this series will be in the range of 200–300 pages, but on occasion a collective work of major importance may be included somewhat exceeding this figure. The intent of the publishers is to bring out these books promptly and in fairly quick succession.

It is on the basis of all these various considerations that we welcome the opportunity of supporting the publication of the series *'Frontiers of Biology'* by North-Holland Publishing Company.

E.L. TATUM
A. NEUBERGER, *Editors*

Preface

Immunosuppressive agents are now widely used in man. They are also utilised in many experimental studies. However, little is known about the mode of action of the few agents used. There is a generally accepted hypothesis according to which the postulated (but often uncertain) biochemical impact may easily explain the immunosuppressive effect. For example, 6-mercaptopurine is supposed to inhibit nucleic acid synthesis and thus inhibit lymphocyte proliferation. The consideration of the biological effect of this drug cannot, however, in our estimation, solely be explained by this simple and a priori logical hypothesis. The mode of action of antilymphocyte serum and of steroids has been the matter of considerable controversy. Finally, explanation of the mode of action of immunosuppressive agents remains essentially open and it is this uncertainty which has justified my effort to review in as analytical a way as possible the mode of action of the four agents most commonly used: 6-mercaptopurine (and its derivative, azathioprine), cyclophosphamide, hydrocortisone (and its derivatives) and antilymphocyte serum. My interest was also roused by the action of immunosuppressants and recent research in cellular immunology, particularly concerning B and T cells. I deliberately left aside other agents, realizing, however, the potential of products such as methylhydrazine, methotrexate and asparaginase. This approach was justified both by the lack of detailed data on the mode of action of these agents, and by their limited use as immunosuppressive agents. The difficulties encountered with the four well-documented products outline the quasi-impossibility of investigating the effects of the lesser-known drugs. Nor have I considered the clinical aspects, as the number of controlled trials have been too few. As a clinical immunologist, my belief is that clinicians will derive more benefit from considering the basic mode of action of the main immunosuppressive

agents, rather than by reviewing the scanty immunological data found in clinical studies. Indeed our conclusions may help the clinician to select and to put to full use immunosuppressive agents, as will be detailed in the concluding chapter. Let me now acknowledge my coworkers Mireille Dardenne and Marie-Anne Bach, together with whom I obtained most of the data (often unpublished) presented in this book, and Professor Jean Hamburger, who guided me in the field of immunosuppression when I entered his renal transplantation unit in 1965. Finally, I should like to thank Claude Ollivier and Catherine Slama, who had the enormous task of typing this manuscript, and Madeleine Vié, who compiled the 1709 references for the bibliography.

Chapter 1

General introduction

The literature on immunosuppressive agents contains several thousand references which I have had no ambition to review extensively. The purpose of this monograph is to examine the data available on the mode of action of the four main classes of immunosuppressive agents: corticosteroids, thiopurines, alkylating agents and antilymphocyte sera. I have deliberately excluded from this study biological or chemical products on which the available information is insufficient to be able to determine whether they are true immunosuppressants or not (see Table 1), and also those products recognized as real immunosuppressive agents but for which the experimental data are too scanty to provide a valuable basis for discussion of the mode of action particularly at the cellular level. Namely, this is the case for amethopterin, methylhydrazine derivatives, cytosine arabinoside and asparaginase.

We have also limited the scope of the review to experimental data associated with the immunosuppressive activity of the products, leaving untreated most of the data dealing, for example, with direct antitumor activity or metabolic effects. Lastly, we have, almost totally, avoided any reference to clinical trials except those including detailed and controlled study of the drug effects on immune responses in man against well-defined antigens. This attitude was justified by the high number of uncontrolled reports, the lack of objective criteria of drug efficiency, the heterogeneity of drug treatment schedules (with often several drugs given in association), and the poor understanding of the immunological background of the diseases considered. These remarks must not be taken as criticisms of these trials and it is recognized that they must indeed be carried out, but our point is that, in the present situation their results do not give much information on the mode of action of immunosuppressive agents, with a few exceptions including organ transplantation which is a relatively well-defined situation.

TABLE 1 Nonexhaustive list of immunosuppressive agents (wide experimental evidence is available for the products underlined)

Purine analogues:	6-mercaptopurine, azathioprine, thioguanine
Alkylating agents:	cyclophosphamide, chlorambucil, melphalan, mechlorethamine, TEM, myleran, nitrogen mustard
Folate analogues:	amethopterin, aminopterin
Pyrimidine analogues:	cytosine arabinoside, BUdR, 5-fluorouracil, 5-fluoro-2'-deoxyuridine
Corticosteroids	
Antibiotics:	mitomycin C, actinomycin, chloramphenicol, azaserine, puromycin, ovalocin, alanosine, adriamycin
Others chemicals:	methylhydrazines promethazine — thalidomide colchicine — chlorphenesine vinblastine — cinaserin vincristine — salicylate ε-aminocaproic acid
Biological agents	antilymphocyte sera antimacrophage sera phytohemagglutinin L-asparaginase ribonuclease polynucleotides
Ionizing radiations	
Miscellaneous	virus, microbial endotoxin, parasites products inducing reticulo–endothelial blockade

Several reviews on immunosuppressive agents have already been published (Hitchings and Elion, 1963; Makinodan et al., 1970; Gabrielsen and Good, 1967; Schwartz, 1965, 1968; Gerebtzoff et al., 1972; Berenbaum, 1965, 1967; Elion and Hitchings, 1965). As mentioned above I have not tried to quote extensively previous works dealing with the effects of immunosuppressants on various types of immune responses. I shall insist rather on the new biochemical and especially immunological data published in the last few years, generally not covered by the previous reviews, and which will cast some light on new aspects of the cellular mode of action of the immunosuppressive agents considered.

I. Problems of definition

Immunosuppressive agents may be defined as products depressing or sup-

pressing immune responses. This definition has the merit of simplicity, but in fact, is very imprecise in view of the high polymorphism and complexity of immune responses.

The knowledge of the mechanism of action of immunosuppressive agents should help in their strict definition as well as in the setting up of adequate screening and evaluation tests. In fact, as will be detailed in this monograph, this knowledge is still very preliminary both at the biochemical and at the cellular level. As will be discussed further, all immunosuppressors do not act identically on T cells, essentially responsible for delayed hypersensitivity and graft rejection, nor on B cells involved in antibody production. Thus, antilymphocyte sera (ALS) and azathioprine seem to have a preferential action on T cells, whereas alkylating agents affect both B and T cells. Moreover, at the cellular level not all immunosuppressors have a similar action. Certain products such as cyclophosphamide are mainly cytolytic, some may be mainly antimitotic such as methotrexate and others promote the elimination of lymphocytes by opsonization in the liver (such as ALS), and finally others may act reversibly on lymphocyte membrane (like azathioprine). It is not surprising that the various categories of immune responses will show different sensitivities to any of these or other immunosuppressive agents. Conversely there are few, if any, immunosuppressors showing maximum activity against all types of immune responses.

These facts call forth several comments: the immunosuppressor definition should allude to the existence of these various categories of immune responses, and hence immunosuppressors may now be defined as '*products able to depress or suppress the development of at least one type of immune reaction*'. This definition is more restrictive than the initial one, where the totality of immune responses was globally considered. This new definition does not, however, solve all problems. Two aspects remain particularly difficult to delineate, (1) the relative effects of the products on the development of the specific lymphocyte sensitization and the effects on the nonspecific expression of this sensitization, in other words, the relative central and peripheral (anti-inflammatory) effects, and (2) the acceptable difference between toxic and active doses or concentrations, a particularly difficult problem in case of low therapeutic index.

II. The problem of the anti-inflammatory effect

Clinical manifestations of immune responses are often the consequence of inflammation. Thus, anti-inflammatory drugs may decrease the expression of

immune responses without modifying the specific immunologic sensitization of lymph·id cells which keep all their pathogenetic potentialities demonstrable, for example, by cellular transfer in untreated animals. This is the case for indomethacin, which is a prostaglandin synthetase inhibitor and which may, under some circumstances, delay skin graft rejection in guinea pigs (Coppola et al., 1970). Conversely, immunosuppressive agents often have anti-inflammatory properties as will be detailed for the four classes of immunosuppressors which are considered in this monograph. This anti-inflammatory action may be very intense in certain cases and does not seem to be always related to the immunosuppressive activity. Anyhow, one conceives how difficult it is to relate the final effect of a given product on immune responses with immunosuppressive rather than anti-inflammatory activity. This is, however, an important question both in the clinical and the experimental use of drugs. Clinically, if presumed immunosuppressive drugs act mainly through their anti-inflammatory activity, it is better instead of these drugs for one to use a true anti-inflammatory compound devoid of the feared side-effects of immunosuppressors, such as bone marrow aplasia and infections.

In experimental studies it may be disturbing to use a drug with a known and postulated biochemical and cellular impact and to have the experiments biased by a secondary effect on the expression of the reactions studied.

While it is difficult in the human to make a difference between immunosuppressive and anti-inflammatory effect, it is often easier in other species by comparing the activity of the product in question administered either during the first days after the antigenic stimulation, at the time of lymphocyte proliferation and sensitization or, conversely, in the few days before reading the reaction, that is to say before the time of rejection or before challenging for delayed hypersensitivity reactions. This approach had been used successfully by several authors (Currey, 1971; Perper et al., 1971).

III. Therapeutic index

The immunosuppressive actions of many compounds are often only obtained at doses close to toxicity. In man, regular clinical and biological surveys must be made in order to gradually decrease in time the dosage in case of side-effects. In view of the relatively small difference between efficiency and toxicity, it is in practice crucial to determine, for each given drug, the best schedule of administration which will give the best immunosuppression with the least toxicity. This research is feasible in the animal but it is more difficult in man to carefully and systematically study the role of the duration of

the treatment, the route of administration, the total dose and also the distribution of doses. The problem is complicated by species variations which prevent an easy extrapolation from one species to another. This is, however, a very important matter since it is, for example, not known in man, for a given total dose and for a similar bone marrow toxicity whether frequent administration of weak doses affords a different immunosuppression than the administration of single doses given at large intervals of time. The availability of new methods to study the level of serum metabolites of most drugs should help in that respect as it will be reported later.

In vitro data is interesting since it provides information on the activity of the product on lymphoid cells independently of the in vivo metabolism, which might induce a too rapid elimination of the product and also independently of extralymphoid toxicity, such as bone marrow toxicity. However, this is an imperfect approach since one may always argue that in vitro data does not necessarily correspond to in vivo activity, in terms of the mode of action of the drug, and moreover some products may only be active in vivo needing metabolic transformation. Lastly, some products may act at a stage of the immune response which is not represented in in vitro reactions, even when using relatively complete immune reactions such as the Mishell and Dutton's technique or the mixed lymphocyte reaction. Finally, some products may show true immunosuppressive activity in vitro at noncytotoxic concentrations but induce no immunosuppression in vivo generally because the serum level of their active metabolites is not sufficient at doses which do not show general toxicity (either because the peak of activity is insufficient or because the duration of high level activity is not long enough). For these products, one should rather speak of potential immunosuppressors and reserve the name of 'immunosuppressor' for products which show significant suppression of the immune response in vivo at nontoxic doses. Finally, one finds again the classical notion of therapeutic index, ratio of toxic doses to efficient doses. In fact, it should be stressed at this stage that the ideal immunosuppressive drug should fulfill the five main requirements (Turk, 1967):

1. There should be a wide margin of safety between the toxic dose and the therapeutic dose.

2. The drug should have a selective effect on the lymphoid cells of the reticuloendothelial system, and not cause damage to the rest of the body.

3. If possible this effect should only be on those cells which are specifically involved in immunological processes.

4. The drug of choice should only need to be administered for a limited period, until such a time as the immunological processes become familiar with

the foreign antigen and begin to recognize it as part of 'self'. After this time it should be possible to reduce the dosage and finally to dispense with the drug so that the animal can maintain its own immunological defences against microbial infections.

5. The drug should be effective against the immune processes once they have already developed.

At the present time none of the available immunosuppressive drugs attains these ideal conditions.

IV. Problems in drug evaluation

The problems of definition of immunosuppressive agents outlined above indicate clearly the difficulties encountered in immunosuppressive drug evaluation and screening. The pharmacologists or the clinicians have to determine whether a given molecule or biological product is immunosuppressive at non-toxic doses. The pharmacologists will be mainly involved in animal studies, and with difficulties in choice of the immunological model as well as in extrapolation to other species. The clinician will ask whether the product known for its immunosuppressive activity in animals will be active in diseases involving immunological phenomena or whether a patient treated by the product is indeed 'immunosuppressed'. These are complex problems to which imperfect solutions can be given and we shall limit ourselves to formulating the questions which should be asked before using a drug as an immunosuppressant clinically (Table 2). We shall also propose two series of simple tests, to be used consequently for the screening or the clinical study of immunosuppressive agents (Tables 3 and 4).

TABLE 2 Five questions to be posed before using a new immunosuppressive drug clinically

1. Has the product been proven to be immunosuppressive in animals and if so in what species?
2. What types of immune responses have been investigated? (see Table 3)
3. What is the therapeutic index? What are the minimal doses giving respectively immunosuppression and toxicity?
4. Are there any data on the best schedule of drug administration (with regard to antigen injection)?
5. Has the immunosuppressive activity been proven in man on antibody production or on cell-mediated immunity independently of any autoimmune responses? (see Table 4). At what dose was the drug given and for how long?

TABLE 3 Screening tests for immunosuppressive agents

A. In vitro tests
(performed directly on lymphocytes from animals treated by the drug or on normal lymphocytes with the original drug or with serum of drug treated animals)

1. Inhibition of spontaneous rosette formation with sheep red cells by mouse and human cells
2. Inhibition of mixed lymphocyte reaction
3. Inhibition of in vitro response to phytohemagglutinin and concanavalin A
4. Inhibition of the primary in vitro immune response to SRBC (Mishell and Dutton's technique)

B. In vivo tests
1. Antibody response to sheep red blood cells
   - humoral antibodies: agglutinins, hemolysins
   - cellular responses
     B cell: ϑ-negative, azathioprine-resistant RFC
     T cell: ϑ-positive, azathioprine-sensitive RFC
2. Antibody response to
   bovine serum albumin (thymus-dependent antigen)
   polyvinyl pyrrolidone (thymus-independent antigen)
3. Cell-mediated immunity
   skin allografts crossing or not the H2 barrier

TABLE 4 Investigation of immunosuppression in man

In vivo testing
humoral antibody responses to
   hemocyanin, influenza (primary and secondary responses)
   tetanus toxoid, poliomyelitis vaccine, vaccine virus (essentially secondary responses)
cell-mediated immune responses
   cutaneous response to tuberculin, varidase, mumps, candidin or trichophyton (established delayed hypersensitivity)
   or to DNCB (induced)

In vitro testing
(performed directly on lymphocytes of the treated patients or on normal lymphocytes incubated with the drug or with serum from treated patients).
in vitro specific response to soluble antigens (PPD...)
mixed lymphocyte reaction
in vitro response to phytohemagglutinin and concanavalin A
rosette formation with sheep erythrocytes (E rosettes)
   antibody-coated erythrocytes (EA rosettes)
   complement-coated erythrocytes (EAC rosettes)
surface immunoglobulin
number of B and T cells evaluated by specific cytotoxic antisera

TABLE 5 Evaluation of ALS immunosuppressive potency (references will be found in chapter 5; ND = not done)

In vivo testing
(in man, or in chimpanzees or macaccus for antihuman ALS)

- prolongation of skin graft survival (Balner)
- depression of delayed hypersensitivity (Balner, Traeger)
- in vivo opsonization (Martin)
- inhibition of local graft-versus-host reaction (Saleh)

In vitro testing (see references in chapter 5, pp. 240-253)

	Correlation with in vivo testing (skin grafts)	
	mouse	human
-rosette inhibition (Bach)	+++	+++
-indirect agglutination (Monaco)	+++	ND
-indirect immunofluorescence (Thomas)	++	++
-complement fixation on platelets (Balfour)	ND	++
-inhibition of mixed lymphocyte reaction (Revillard)	ND	++
-opsonization (Greaves, M.K. Bach)	+++	–
-cytotoxicity	–	+
-agglutination	–	+/–
-inhibition of lymphocytotoxicity		
in presence of PHA (Holm, Moller)	ND	+
against antibody-coated target cells (Holm)	ND	ND

When biological products are considered such as antilymphocyte serum or ribonuclease, question 5 must be reexamined for each batch of product, and evaluation of immunosuppressive potency becomes then a crucial problem for clinical use. Thus for ALS numerous methods listed in Table 5, have been proposed. The consensus seems now to do the screening by the rosette inhibition test which is easy to perform and does not consume much material. Correlation of its results with immunosuppressive potency is documented by numerous studies (see pp. 240–253) both in the mouse, the dog and the human. When large batches are ready to use clinically, it is probably useful to verify their activity (and absence of toxicity) in the monkey. New in vitro tests have recently been claimed to be as informative as the rosette inhibition test such as the indirect immunofluorescence test, indirect agglutination or complement fixation on platelet. Not enough data is available to make a clear judgment on these promising methods.

V. The main experimental approaches

The difficulty in studying the mode of action of pharmacological agents in general and immunosuppressive agents in particular, is that even when a precise locus of action is determined, it is always impossible to know if it is the major mode of action of the drug or whether there are other impacts which may be relevant to the biological effect. This is true both at the biochemical and cytological level and it explains why no definitive conclusions will be proposed for any of the four classes of products considered. One may distinguish several levels of investigation of an immunosuppressive agent, which will be systematically examined further for corticosteroids, thiopurines, alkylating agents and antilymphocyte sera.

1. Biochemistry

The biochemical lesions induced by the drug are certainly of primary importance and in fact it is on the basis of these biochemical lesions that the products, at least the chemicals, are generally conceived. It is of importance to determine the various impacts of each given drug, as well as the metabolic transformations undergone by the drug and the listing of the various metabolites produced.

2. Pharmacology

Pharmacological studies have first to determine the acute or chronic toxicity, which is a major requirement for immunosuppressive agents which are often only active as immunosuppressants at doses not far from toxicity. The drug metabolism or, more precisely, the level of active metabolites in the serum, and the drug distribution in the organism should be determined accurately. The mode of transport of the drug is also of importance. It is very difficult and sometimes impossible to determine what is the mode of action of a given product without knowing the kinetics of active metabolites.

3. Actions at the subcellular level

It is interesting to know if the products examined induce changes in the cell metabolism, including changes in protein synthesis or DNA synthesis particularly at the level of lymphoid cells and macrophages. It will be also important of course to determine whether the drug may have, under certain circumstances at least, an antimitotic effect and whether it may induce cell death after incubation in vitro. More generally it is important to determine at what stage of the cell cycle the target cell is the most sensitive to the drug: S phase (DNA synthesis), G2 phase (postsynthetic phase), M phase (mitosis) or G1

phase (interphase). The impairment of cell reproductive integrity may indeed be one of the essential modes of action of several immunosuppressive drugs as indicated by the correlation of immunosuppressive and antitumor activity, time-dependent effects and known biochemical impact (Berenbaum, 1970).

Indirect information on the subcellular mode of action may be obtained by consideration of the dose–effect curves of single doses (Berenbaum, 1967). Thus, irradiation and alkylating agents induce lesions which do not interfere between each other (as eggs destroyed by randomly thrown objects) which explain their experimental dose–effect curve. Conversely thiopurines or methotrexate which interact mainly with enzymes alter the probability of other reactions and have hyperbolic dose–effect curves the product of dose and of surviving cells being constant.

4. Actions on cellular functions

It is an important advantage of immunology to allow the study of cellular function at cell level (Table 6). This is generally impossible for other disciplines where most functions can only be studied at the organ level. It is possible with most immunosuppressive agents, as will be detailed in the following pages using this advantage, to determine what are the actions of the agent in question on the multiple cellular lymphoid cells and macrophages. We shall not consider here the (sometimes controversial) significance of each of the in vitro tests listed in Table 6 which will be found in the corresponding references given in the table. Let us, however, mention that the battery of all these tests allows a very refined investigation of all steps of the immune response (1) antigen processing (phagocytosis), (2) antigen binding by T and B lymphocytes (RFC), (3) differentiation and proliferation of antigen-sensitive cells (DNA synthesis in the presence of antigen and mixed lymphocyte reaction), (4) antibody synthesis (Mishell and Dutton's technique), (5) antibody release (PFC and RFC), (6) lymphokine release (MIF assay), (7) lymphocytotoxity (Brunner's assay, CML).

5. Search for a selective cell depletion

As many immunosuppressive agents induce depletion of some lymphoid cells it is of importance to determine whether this depletion is selective for one given lymphocyte population. This may be approached either by counting lymphoid cells in various lymphoid organs, looking at the proportion of cells bearing various markers or by looking at the histopathology of lymphoid organs (Table 7). It is also possible to look at the percentage of cells with long or short life span or of recirculating cells using various radioactive labels (^{51}Cr, tritiated thymidine, IUdR).