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IMMUNOSUPPRESSIVE DRUGS[1]

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I. INTRODUCTION

Dramatic clinical successes in some organ transplantations have stimulated a great deal of interest in control of the immune response for therapeutic purposes,

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not only in the field of transplantation but also in the area of autoimmune diseases. Methods of immunosuppression are still imperfect and the value of widespread clinical application remains uncertain. Thus a vast amount of work is still to be done before the ultimate objective will be attained in the field of transplantation; for example, there is a need for a method to rapidly and specifically induce and, if necessary, to terminate immunological tolerance, without causing irreparable damage to the immunological and other vital tissues of the recipient.

Studies on experimental alteration of the immune response were initiated early in this century, especially by those concerned with the effects of X-irradiation (29, 188, 237). About 15 years ago there began a surge of interest in immunosuppressive agents resulting from several major discoveries in experimental and clinical immunology. They were as follows: 1) the ability to induce in adults specific unresponsiveness to an otherwise effective antigen (108, 184); 2) the ability to perform long-term transplantation of organs that normally would be rejected within a short time because of the genetic disparity between donors and hosts (288, 334); and 3) the recognition that drugs may be used to combat autoimmune diseases (154, 373).

Our survey will be restricted primarily to the effect of immunosuppressive drugs on humoral and cell-mediated immune responses. Therefore, we are excluding immunosuppression by antigen competition, by infusion of specific antibody, antilymphocyte reagent, or α_2-globulin fraction, by organ extirpation, by thoracic duct drainage, and by exposure to whole-body, local, or extracorporeal ionizing radiation.

We will first present a view of the mechanism of immune response at the cellular level in order to emphasize the various events that may be susceptible to the drugs. Then we will briefly discuss immunological tolerance, as it is the ultimate objective of immunosuppression. Since the field has been expanding so rapidly in the past few years, and there has been such lack of uniformity of approach to the study of immunosuppression that it is difficult to interpret the existing data in a meaningful way, we will give preference to drugs that show clinical potential or afford insight into the nature of immune responses. The appendix contains tables with information about relative effectiveness, dose, type of recipient, and key references of the various classes of drugs.

II. THE IMMUNE RESPONSE

A. General description

All vertebrates except possibly the lowest forms respond adaptively to foreign substances found in parasitic organisms, tissues of other species, and tissues of other organisms of the same species. The stimulating foreign substances are called *antigens* or *immunogens*, and the adaptive response, which involves cells of the lymphoreticular system, *immune response*. In an immune response most of the antigens become coated with a complex mixture of proteins called *opsonin*, and opsonized antigens are generally engulfed and digested by scavenger cells called *phagocytes*. However, some are "processed" by *macrophages* or *macrophage-*

like cells; these cells initiate a series of cellular events leading to the appearance of terminal effector cells that can either destroy the complex antigen-carrier upon direct contact, as in the case of transplanted living foreign cells, or synthesize and secrete proteins known collectively as *immunoglobulins*, varying in molecular weight from about 150,000 (7 Svedberg units or 7 S) to about 1,000,000 (19 S). The immunoglobulins that can react specifically to the stimulating antigen are called *antibodies*.

Complexes resulting from the binding of antigens by the antibodies can, in turn, bind and activate a group of proteins known collectively as *complement*. Most such complexes are rapidly catabolized. In certain instances such complexes can lead to inflammation-inducing events, for example, *chemotaxis* and *anaphylaxis*. In other instances the complexes can circulate and eventually be deposited on tissues, leading to inflammatory changes in the tissues, as in some cases of *glomerulonephritis* (431a). When the antigen is contained in a living foreign cell, antibodies that do not bind all of the complement components can shield this cell from the host cells that destroy foreign cells and from complement-binding antibodies. This shielding is called *immunological enhancement*.

When an organism undergoes an immune response for the first time it is called a *primary* response, and generally little antibody is produced. When the organism is exposed to an antigen for the second or subsequent time, the response is more rapid, its magnitude more pronounced, and its duration much longer than in the primary response. It is called the *secondary* or *anamnestic* response. When the first exposure occurs under certain conditions, the organism may not respond to an antigen. In this case the antigen is handled as if it were a component of the host. This is known as *immunological tolerance* or *paralysis* (section III).

B. Model experimental systems

For proper analysis of the effect of drugs or other insults on the immune response it is important to have at hand some information concerning the kinetics of the immune response in an unperturbed test system. Our present understanding of the cellular kinetics of the immune response is based largely on studies *in vivo* (267, 388), and more recently also studies *in vitro* (111). In the former studies, highly inbred mice have been the choice experimental animals. The procedures involve (a) suppression of the immune function of the prospective recipient mice by exposing them to ionizing radiation (266), by treating them with immunosuppressive drugs (364), or by a combination of surgical thymectomy and X-irradiation (87, 98, 293), (b) either infusing into these mice a mixture of the test antigen at varying concentrations with various immunocompetent cells in varying numbers—the cell transfer method—or by placing this mixture of antigen and immunocompetent cells into a chamber constructed of a Lucite ring and two cell-impermeable membranes and then placing this chamber into the peritoneal cavity of the recipient—the diffusion chamber technique—and (c) assessment of the immune response generated by the donor cells by measuring the number of antibody-producing cells present at various times.

Because many of the detailed, quantitative studies of effector cells have been

based on the response mediated by circulating antibodies, emphasis will be placed on the formation of antibody-synthesizing cells. Less known are the antigen-triggered events leading to the formation of effector cells capable of graft rejection and delayed hypersensitivity; however, they probably differ at most only quantitatively from those involved in formation of humoral antibody (18, 243).

The production of serum antibody in an organism can be divided into 4 distinct sequential phases (lag, log, plateau, and decline phases). The lag phase is the interval between time of antigen injection and beginning of the exponential rise in antibody concentration in the blood. This phase usually extends from one to a few days. The log phase is the interval in which antibodies are released exponentially into the blood so rapidly that the concentration doubles about every 8 hr (*e.g.*, see 9). This phase generally lasts 2 to 4 days. The postlog phases of serum antibody response vary immensely and depend primarily on the type and dose of antigen and the quality of antibody released into the blood. When young adult mice are stimulated intravenously with an optimal dose of sheep red blood cells, for example, the plateau phase of hemolysin response (immunoglobulin M) is absent and its decline is short, as would be expected if no antibody were being released into the blood (218). However, the plateau of agglutinin response (immunoglobulin G) is extended for many days; this is followed by a decline that can extend for many months (9). During the plateau the rate of release of antibody into the blood is the same as the rate of elimination of antibody from the blood (344). Hence this phase has also been called the "steady-state" phase.

C. Genesis of antibody-synthesizing cells

The genesis of immunocompetent cells, cells that can respond to antigenic stimulation, is not thoroughly understood. The current data suggest that in mammals the precursors of immunocompetent cells are the lymphohematopoietic stem cells (fig. 1, left), which have the potential to differentiate into many types of functional cell (138, 423). The data further suggest that the primordial stem cells originate in the yolk sac (300, 427, 428). In young adulthood most of the stem cells reside in the bone marrow and emigrate from there by way of the blood and lymph to various organs (159, 181, 292). Some migrate directly into the spleen, lymph nodes, and peritoneal cavity, and these cells can be called *bone marrow-derived* cells. Others migrate into the thymus, and then from the thymus to other organs and tissues, including the spleen, lymph nodes, and peritoneal cavity. Conceivably some may even return to the bone marrow. These cells can be called *thymus-derived* or *thymus-influenced* cells.

Claman *et al.* (87) were probably the first to demonstrate definitively that the interaction of these two cell types was essential for the initiation of an antibody response. The basis of their study lies in the earlier work of Fishman (131, 132), who showed that both lymph node cells and macrophages or macrophage-like cells of the peritoneal fluid are required for the initiation of antibody response to a particulate antigen. Subsequently, Davies *et al.* (98, 99), Miller and Mitchell and their colleagues (277, 293, 294, 314), and Mosier *et al.* (303–305) showed

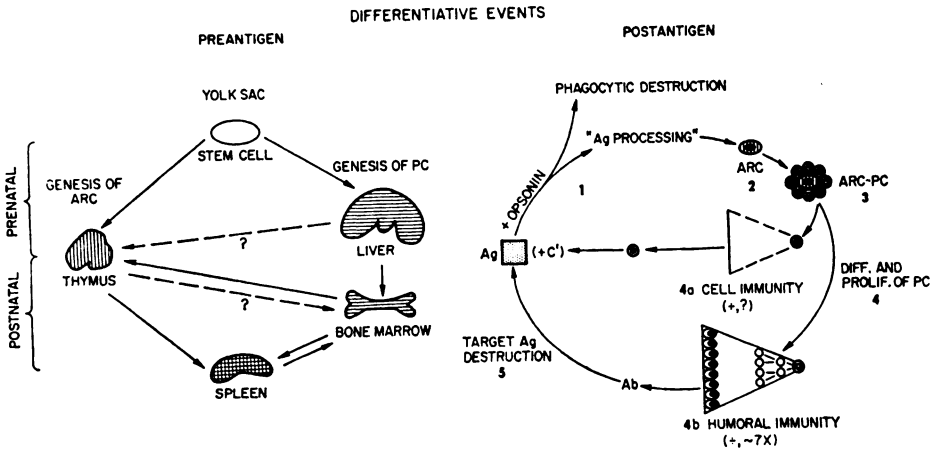


FIG. 1. A model of differentiation within the immune system. Arrow indicates direction of differentiation of stem cells. Ag, antigen; Ab, antibody; PC, precursors of antibody-synthesizing cells; ARC, antigen-reactive cells; Diff., differentiation; Prolif., proliferation; C', complement; ÷, mitotic division.

that the events after antigenic stimulation occur most probably in the following sequence (fig. 1, right):

1) The antigen reacts with the thymus-derived cells. Hence, these are also called *antigen-reactive* cells (ARC, fig. 1, right). It is possible that the antigen-reactive cells are the same cells as the "antigen-processing" and "glass-adhering" cells of the peritoneal fluid and the spleen, but definitive evidence for this is lacking. The latter cells possess properties of a macrophage and "process" antigen for the initiation of antibody response (fig. 1, right) (131, 299, 303). For the sake of simplicity we are assuming that these cells and the "antigen-trapping" cells of the lymph follicle (3, 449) are identical, and the term antigen-reactive cells will be used in this review.

2) The bone marrow-derived cells then make contact with the antigen-reactive cells that have reacted with the antigen (fig. 1, right).

3) In response to antigen stimulation, by some unknown mechanism, the bone marrow-derived cells undergo transformation and proliferation, giving rise to many antibody-synthesizing cells (fig. 1, right). Hence the bone marrow-derived cells have often been called the *precursor cells* (PC fig. 1) of antibody-synthesizing cells. Thus, in a typical antibody response one can envision the existence of immunocompetent units or "functional clones" of varying sizes in the spleen and lymph nodes, analogous to clones of cells observed in tissue culture.

It should be noted that theories have been proposed (305, 416) requiring the interaction of 3 cell types for the initiation of a primary antibody response. However, because the current quantitative data are explicable in terms of 2-cell interactions (173, 174), there is no urgent need to abandon the 2-cell theory.

It would appear that each immunocompetent clone can respond to only one

antigen (227). However, we do not know if this specificity arises in antigen-reactive cells, precursor cells, or both. The significance of this restrictive potential of antigen-stimulable immunocompetent clones will be discussed under drug-induced tolerance (section V): by judicious use of certain drugs together with an antigen, it is possible to "wipe out" those clones which are responsive only to the injected antigen.

D. Cellular kinetics

The interaction between antigen-reactive cells and precursor cells may be approached through quantitative studies of 1) limiting dilution and 2) dose-response. The former type of study measures the number of functional clones involved in initiating an antibody response and involves transferring small and varying numbers of donor immunocompetent cells into a recipient (as outlined by either of the 2 methods described in section B above) with a constant amount of antigen and then, several days later, measuring the number of antibody-producing cells generated. The latter type of study measures the *magnitude* of the antibody response and involves transferring varying numbers of immunocompetent cells into a recipient while keeping the amount of antigen transferred constant and then, several days later, measuring the number of antibody-producing cells generated. Recently, Groves *et al.* (173, 174) measured the primary antibody response of dispersed mouse spleen cells against sheep red blood cells in cell-impermeable millipore diffusion chambers. Limiting dilution analysis demonstrated that the number of clones was a linear function of the dose of spleen cells. The logarithmic dose-response curves were biphasic: at low doses of spleen cells the quantity of antibody produced was a nonlinear function of the dose, but this relationship abruptly became linear at higher doses. On the basis of these and other results Groves *et al.* concluded that each antigen-reactive cell can accept up to about 8 precursor cells, provided that each precursor cell upon stimulation divides about 7 times. This means that the initial size of an immunocompetent clone can vary from 2 cells (1 antigen-reactive cell and 1 precursor cell) to 9 cells (1 antigen-reactive cell and 8 precursor cells). They further concluded that in a typical immune response there is always an excess of precursor cells and that therefore in the intact animal all the precursor-cell-receptor sites of most antigen-reactive cells are usually saturated with precursor cells. This means that in a typical primary response each immunocompetent clone, made up initially of a rosette containing 9 cells, can generate about 1000 antibody-synthesizing cells, *i.e.*, 8 precursor cells/clone X 128 progenies/precursor cell (1 cell dividing 7 times results in 2^7 or 128 progenies) (see fig. 1).

In a primary response about 1 immunocompetent clone in 100,000 spleen cells is responsive to a naturally occurring, complex antigen (9, 54, 70, 226, 370, 386). This means that at the height of response 100 to 1000 out of 100,000 spleen cells (0.1 to 1.0%) should be synthesizing antibody, and this ratio of responding cells has been observed (114, 218, 242, 436, 455). In the spleen of an organism that has been previously immunized there can be 10 to 100 times more precursor cells than in the spleen of an unimmunized organism (9, 264,

330, 387). However, because of restrictions imposed by homeostatic forces, including possibly some kind of feedback regulation perhaps influenced by antibody concentration, only a small fraction of the total population of cells responsive to the test antigen undergo a secondary response at any given time (264, 330). Thus, for example, at the height of a secondary response, far less than the expected 10% of the spleen cells are synthesizing antibody (54, 114, 242, 436, 455).

A primary response is generally characterized by a wave of cells synthesizing 19 S immunoglobulin antibodies, followed by a wave of cells synthesizing 7 S immunoglobulin antibodies, which makes its appearance at the crest of the first wave (*e.g.*, see 455). One possible explanation for the disappearance of 19 S antibody-synthesizing cells in association with the appearance of 7 S antibody-synthesizing cells is that 7 S antibody may complex and neutralize persisting antigens that otherwise would stimulate potential 19 S immunocompetent clones (430) (see section IV F for the differential action of immunosuppressive drugs). In a secondary response the 7 S antibody-synthesizing cells appear at about the same time as the 19 S antibody-synthesizing cells, but the magnitude of increase of the former is higher (about 10 times) than the latter.

Recently, Perkins *et al.* (327) performed the most comprehensive kinetic study of a primary response to date by assessing at 2-hr intervals the number of 19 S antibody-synthesizing cells in the spleens of mice; the study was continued until the 4th day, when the response reached its maximum. On the 1st day the antibody-synthesizing cell population began to increase above the background level exponentially in a stairstep manner. This pattern of growth continued until the peak level was attained on day 4, when the population began to decrease drastically. The mean time for the population to double its size during the growth phase was 6 hr. If the amplitude of increase between each stairstep had been 2-fold, this would have indicated that there was a single burst of cells being transformed into antibody-synthesizing cells and that division of immature antibody-synthesizing cells is the *primary*, if not the *sole*, cause for the increase in the population (341). However, the amplitude of increase was 2.5- to 6-fold. This suggests that additional cells were being recruited into the production of antibody-synthesizing cells.

The interval between steps (*i.e.*, the shelf time) was relatively constant and about 9 hr. From these results, results of studies of DNA metabolism and mitosis, and the assumption that the shelf time reflects the generation time of the proliferating cells (344), Perkins *et al.* proposed the following underlying mechanism for the cause of the growth pattern. Precursor cells are normally in resting (G_0) stage. Upon stimulation by antigen-reactive cells they enter cell cycle and divide synchronously; after mitosis some cells become immature, proliferating, antibody-synthesizing cells. The number of such cells increases exponentially after each successive mitotic event. A schematic growth chart is shown in table 1. We know of no other system where growth of a population of cells due to proliferation and recruitment occurs synchronously for such an extended period of time (3 to 4 days).

TABLE 1

*A model for nonrandom multiple recruitment of precursor cells into the antibody-synthesizing cell populations dividing synchronously**

	Time in Number of 9-Hr Generations							
	1	2	3	4	5	6	7	8
	Number of antibody-synthesizing cells							
	1	2	4	8	16	32	64	128
		1	2	4	8	16	32	64
			2	4	8	16	32	64
				6	12	24	48	96
					20	40	80	160
						52	104	208
							160	320
								450
Total	1	3	8	22	64	180	520	1490

* The model represents a case in which recruitment of precursor cells into the compartment of antibody-synthesizing cells is occurring at the time of mitosis of cells with a generation time of 9 hr and in which it takes the population 6 hr to double its size. Growth through division occurs from left to right. The numbers below the diagonal line represent the number of cells recruited from the precursor cell compartment into the antibody-synthesizing cell compartment.

E. Antibody-synthesizing cells

Morphologically, antibody-synthesizing cells constitute a heterogeneous population. Most are plasma cells (23, 26, 101, 432, 436, 449), some are lymphocytes (183, 436), and a few possess macrophage-like features (75). Only the plasma cells possess structural characteristics of efficient protein-synthesizing and -secreting cells, *i.e.*, cytoplasm filled with ribosome-associated endoplasmic reticulum and with a prominent Golgi complex (241). There are two types of plasma cell in terms of proliferative potential, those with the capacity to synthesize DNA and undergo mitosis and those lacking the capacity to proliferate (263, 295, 372).

The ultimate fate of antibody-synthesizing cells is not fully understood; they must either dedifferentiate into nonfunctional cells or die (437, 451). Much of the current data favors the latter hypothesis (124, 343, 410). If death is their ultimate fate, then the path of differentiation of immunocompetent cells is unidirectional and irreversible (9, 437).

Studies on intracellular synthesis of immunoglobulin have been based primarily on the synthesis of 7 S immunoglobulin G by myeloma and normal plasma cells (227). Immunoglobulin G is made up of two pairs of peptide chains held together by disulfide linkage. The larger chain is made up of about 450 amino acid residues and is commonly called the heavy or H chain, and the smaller about 200 acid

residues and called the light or L chain. The two chains are synthesized by separate polyribosomes located on the endoplasmic reticulum (16, 17, 100, 383, 450). The polyribosomes synthesizing the light chains are characterized by having 7 to 8 ribosomes with a combined S value of 150, and those synthesizing the heavy chains by having 16 to 18 ribosomes with a combined S value of 300. Light chains released from 150 S polyribosomes may associate with the heavy chain-synthesizing 300 S polyribosomes, where the assembly of the molecule is initiated. Our knowledge of the mechanisms of the assembly of chains into molecules and their secretion is still incomplete. In most tissues there is an overall balanced production of both chains. However, imbalance occurs in certain myeloma plasma cells to the extent that there is an overproduction of light chains or heavy chains, leading to their secretion into the blood, and, in the case of light chains, also excretion into the urine in fragmented, monomeric, and polymeric forms (Bence-Jones proteins) (44, 393).

Information about the events of differentiation occurring at the nuclear level is almost nonexistent in comparison with that at the cytoplasmic level. At the cytoplasmic level it is well documented that most, if not all, individual antibody-synthesizing cells synthesize and secrete only one discrete type of antibody molecule, as judged by the specificity, class, and allotype of immunoglobulin (79, 82, 172, 287, 328). However, at the nuclear level we do not know what, when, and how the transformation occurs from a multipotential stem cell to a unipotential functional antibody-synthesizing cell. Thus, for example, we do not know which of the structural genes is turned off irreversibly before, and which after, exposure to the antigen.

A demonstration of the hardiness of *mature* antibody-synthesizing cells is that their antibody-synthesizing and -secreting capacities cannot be altered by very high doses of ionizing radiation (265, 343, 434). After exposure to 10,000 r of X-irradiation they can survive for 4 or more days with undiminished rates of synthesis and secretion of antibody. These results emphasize the stability of the peptide-synthesizing polyribosome system, which includes the messenger RNA, as well as the enzymes required for the synthesis and secretion of the antibody.

F. Summary

The evidence indicates that at least two cell types are required for the antigen-induced initiating events leading to the generation of antibody-synthesizing cells. This may also be the case for cells involved in cell-mediated immune reactions. The two cell types are thymus-derived antigen-reactive ("processing") cells and the bone-marrow-derived precursor cells. The actual mechanism for their interaction remains to be determined. However, it is clear that in both primary and secondary responses precursor cells proliferate and become mature, nonproliferating antibody-synthesizing cells, whose ultimate fate is most probably death. Thus, it is possible to interfere with an immune response at several post-antigen events (1 through 5, as depicted in fig. 1).

Theoretically, events involving the antigen-reactive cells can be suppressed by (a) destroying the cells, (b) neutralizing their ability to react with the antigen,

and (c) neutralizing their ability to react with the precursor cells. Theoretically, events involving the precursor cells can be suppressed by (a) destroying the cells, (b) neutralizing their ability to react with the antigen-reactive cells, (c) neutralizing their ability and the ability of their immediate progeny to proliferate, (d) neutralizing the ability of their progeny to synthesize at least one of the two peptide chains, (e) neutralizing the ability of their progeny to secrete antibody, and (f) shortening the life span of their terminal progeny. Finally, it is conceivable that one could reduce the effect of an immune response (see event 5 of fig. 1) by promoting a faster elimination of the antibody and other essential proteins (*e.g.*, by filtration of the shunted blood through a column of immunoadsorbent).

The most difficult phase of antibody response to suppress is the postlog growth phase involving the terminal antibody-synthesizing cells. Two reasons for the difficulty are: (a) antibody-synthesizing cells are the end product of a 1000-fold or greater amplification of an antigen-triggered cellular event, and (b) these cells are very resistant to injury. For these reasons, in order to develop methods of immunosuppression, emphasis should be placed on interference with the antigen-reactive and precursor cells.

III. ACQUIRED IMMUNOLOGICAL TOLERANCE

As a consequence of exposure to an antigen under certain conditions, an organism may not respond to it specifically. This state is known as acquired immunological *tolerance*, *paralysis*, or *unresponsiveness*. There are two compelling reasons for the interest created by this phenomenon. One is that the mechanism of induction of tolerance may play a major role in the prevention of autoimmune reactivity (51). The second, as stated earlier, is that immunological tolerance is the ultimate objective in the field of tissue transplantation. In contrast to the vital damage to both the immunological and nonimmunological tissues caused by the current immunosuppressive methods, induction of immunological tolerance presumably causes a deletion of the immunological reactivity against only the test antigen (and not against other antigens).

This phenomenon came into focus after the study of Billingham *et al.* (50) in 1953, although it had been observed earlier (81, 125, 163, 322, 422). Billingham *et al.* artificially created blood chimeras (individuals possessing donor blood cells) in neonatal mice by infusing into them lymphohematopoietic cells from genetically incompatible (allogeneic) mice. When these mice reached young adulthood they were able to reject skin grafts from all allogeneic donors, except from those which were genetically identical to the donors of lymphohematopoietic cells. This type of tolerance is unusual in having immunocompetent cells of donor origin; in the conventional type the immunocompetent cells are those of the host. The field of transplantation immunology has since blossomed into a major discipline.

It is now possible to induce immunological tolerance to soluble antigens with relative ease in nonimmunized adults and, to a lesser extent, even in previously immunized adults (108). Success is greatly dependent on the dose of the antigen,

its physical state, and the immunological state of the individual. Mitchison (298, 299) emphasized that with highly immunogenic antigens only high doses, as first reported by Glenny and Hopkins (163), induce immunological tolerance. However, with weakly immunogenic antigens there are two effective antigen dose ranges, one lying below and the other above the antigen dose range that can induce an immune response. The importance of the physical state of the antigen was first shown dramatically by Dresser (107). He induced immunological tolerance in mice with unaggregated immunoglobulin G. The tolerance-inducing antigen was prepared by centrifugally removing the highly immunogenic aggregates that are normally present in a preparation of immunoglobulin solution. Other examples of the effectiveness of removal of immunogenic components of antigen preparation have been reported (27, 86, 143).

The importance of the immunological state of an adult has been well demonstrated by the use of drugs. Studies in this area began with the work of Schwartz and Dameshek (377) in 1959; their work will be discussed later (see section V). The basis of drug-induced immunological tolerance can be traced to the earlier studies of Main and Prehn (261), who used ionizing radiation to lower the level of immune competence of adult mice and then transplanted allogenic bone marrow cells that normally would have been rejected.

Reconstitution studies *in vivo* have shown that the lack of responsiveness during tolerance is due to failure among both antigen-reactive cells and precursor cells, depending upon the test system (108). The cellular mechanism of induction of immunological tolerance is poorly understood. That is, we do not know whether induction of tolerance in the case of the competent precursor cell involves death or irreversible inactivation. If the latter is the case, we do not know if inactivation involves failure in one or more of the following possible capabilities: (a) recognition of the "processed" antigen; (b) recognition of the antigen-activated antigen-reactive cell; (c) differentiation into functional effector cells; and (d) proliferation. In view of these considerations and in view of our current understanding of the cellular mechanism of immune response, it is reasonable to assume that within an organism undergoing an immune response there is a wide spectrum of responses occurring among the various antigen-stimulated immunocompetent clones. Some may die, others differentiate but not proliferate, and still others differentiate and proliferate maximally, depending upon the concentration and physical nature of the local antigen. Suffice it here to say that many of these possibilities are now testable.

IV. THE SUPPRESSIVE EFFECT OF CHEMICAL AGENTS ON THE IMMUNE RESPONSE

A. Objective and scope

The existing data on drug-induced immunosuppression are somewhat confusing, partly because of the great number of papers on the subject, but more importantly because of the lack of uniformity in approach to the study of immunosuppression. Very often, reports concerning a given agent are discordant. Examination of individual protocols strongly suggests that the apparent dis-

crepancies could well be related to variables such as species and condition of animals used, dose, dose schedule, route and vehicle of drug administration, type and amount of antigen used, and method of immunological assay. Furthermore, there has often been a tendency to theorize in molecular terms the immunosuppressive action of certain drugs. This would seem quite premature, since most, if not all, of the agents have multiple sites of action on the complex series of cellular and biochemical events that constitute the immune response.

Recently, extensive reviews of immunosuppressive drugs have been published (34, 37, 38, 71, 84, 116, 154, 197–199, 262, 373, 375, 376). In most reviews, the agents were discussed in categories related to their presumed major biochemical activity, such as alkylating agents, *etc.* It has been apparent for some time, however, that two agents of the same presumed class might have quite different effects, operationally at least, on immunological events. This does not imply that the presumed biochemical mechanisms are necessarily incorrect, but that they may reflect differences in metabolism, tissue distribution (245), or cell permeability or, more simply stated, the differences in "whole animal pharmacology."

Notwithstanding the inadequacies in our knowledge, several broad operational principles have become apparent regarding the effects of chemical agents upon immune processes in animals and man. Furthermore, some of these principles have proved quite useful in making clinical as well as laboratory application of several of the agents.

This part of the review will be limited in general to agents about which enough is known or implied that certain operational statements may be made.

B. Timing of drug administration in relation to the antigenic stimulus

In the first part of this review, the cellular and kinetic events of the immune response were outlined and illustrated. It should be stressed that this was a simplified account in that the actual cellular and biochemical events of the immune response are complicated and poorly understood. It would be indeed surprising, therefore, if all the agents in current use affected the immune response in the same way. Because of the varied nature of the chemical agents themselves and their presumed mechanisms of action, one would expect *a priori* that different stages of the various immune response (*e.g.*, antibody formation, delayed hypersensitivity, skin graft rejection) would show differences in sensitivities to the action of various agents. It has been useful to classify the various agents as to the stage at which the immune response in question is more sensitive to their immunosuppressive action.

Class I agents are most effective in suppressing an immune response when given just before the antigenic stimulus and are relatively ineffective when given after. The very early processes of the immune response on which these agents are assumed to act include antigen processing and early "information" transfer.

Class II agents are most effective as immunosuppressants when given a day or two after the antigenic stimulus. The period of mawimal sensitivity may last

a day or two. In general, the cellular proliferation and differentiation of the immune response are more sensitive than other stages of the immune response to the action of these compounds. Furthermore, they are quite ineffective as immunosuppressants when given before the antigen; indeed, some of the agents may enhance the immune response under these conditions. The majority of immunosuppressive drugs are in this class.

Class III agents comprise the smallest group of drugs. They appear to be immunosuppressants whether applied solely before the antigenic stimulus or solely after the stimulus, and thus appear to possess the properties of both class I and class II agents. The agents considered in each of these operational classes are listed in table 2. In table 3 can be found structural formulas for the common immunosuppressive agents.

C. Class I agents

1. Alkylating drugs. Despite the number of papers devoted to the subject, few studies have defined the relationships between administration of alkylating agents and the immune response. Gabrielson and Good (154) have summarized much of the older work with alkylating agents, wherein the data suggested that most of such drugs might be class I agents. The time relationship between immunization and drug administration in most studies, however, was not clear enough to indicate when the immune response was most sensitive to these agents. In order to clarify the functional classification of alkylating agents both class I and II alkylating agents will be discussed in this section.

Alkylating agents are compounds that interact with the nucleophilic centers of other molecules. The most favorable sites of reaction should be molecules possessing $-NH_2$, $-COOH$, $-SH$, and $-PO_3H_2$ groups and those possessing tertiary nitrogen compounds in heterocyclic systems (339). The most common biological components that possess such reactive groups are DNA, RNA, enzymes, structural proteins, and cell-wall constituents. There is experimental evidence that mechlorethamine reacts preferentially with the 7 position of guanine in DNA (239), and that difunctional nitrogen mustards cause cross-linkages in DNA (67). Other sites that may be alkylated are the phosphate groups in DNA and RNA, but alkylation here occurs only to a very small extent (238). The acidic and basic groups of proteins could also be sites of action for this group of compounds. It is possible to esterify the carboxyl groups in native proteins, but the amino groups can be attacked only by the epoxides and by none of the other alkylating agents (398).

The alkylating agent L-phenylalanine mustard has recently become of clinical interest because of its therapeutic effect on plasma cell malignancy (48, 320). When a single injection of L-phenylalanine mustard was given to mice at various times in relation to the injection of sheep red blood cells and agglutinin titers were determined 7 days after immunization, suppression of the antibody response was greatest when the drug was given 1 to 2 days before immunization (69). Nevertheless, some effect occurred if the compound was given a day or two after immunization.

TABLE 2

Operational classification of some immunosuppressive agents

Class	Official or Generic Name	Chemical Name	Trade Name	Common Name
I (active primarily when given before immune stimulus)*	Busulfan	1,4-Butanediol dimethylsulfonate	Myleran®	
	Colchicine			
	Cortisone	17α,21-Dihydroxy-4 pregnene-3,11,20-trione		
	Medphalan	D-3-{*p*-[Bis(2-chloroethyl)amino]}-phenylalanine		D-Phenylalanine mustard
	Melphalan	L-3-{*p*-[Bis(2-chloroethyl)amino]}-phenylalanine	Alkeran®	L-Phenylalanine mustard
	Mitomycin C			
	Phytohemagglutinin (PHA)			
	Prednisolone	11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione		
	Prednisone	17α,21-Dihydroxypregna-1,4-diene-3,11,20-trione		
II (active primarily when given after immune stimulus)	Aminopterin	N-{*p*[2,4-Diamino-6-pteridyl-methyl)amino]benzoyl}glutamic acid		4-Amino folic acid
	Ara-cytidine (Ara-C)	1-β-D-Arabinofuranosylcytosine	Cytarabine®	Cytosine arabinoside
	Azathioprine	6-[(1-Methyl-4-nitroimidazol-5-yl)-thio]purine	Imuran®	
	5-Bromo-2′-deoxyuridine (5-BUdR)			
	Chlorambucil	4-{*p*-[Bis(2-chloroethyl)amino]-phenyl}butyric acid	Leukeran®	
	Chloramphenicol		Chloromycetin®	
	5-Fluoro-2′-deoxyuridine (5FUdR)			
	5-Fluorouracil (5FU)			
	Mechlorethamine [NH2]	2,2′-Dichloro-N-methyldiethyl-amine	Mustargen®	Nitrogen mustard
	6-Mercaptopurine (6-MP)		Purinethiol®	
	Methotrexate	N-{*p*-[2,4-Diamino-6-pteridyl-methyl)amino]benzoyl}glutamic acid	Amethopterin®	4-Amino-10-methyl folic acid

	Thioguanine (TG)	2-Aminopurine-6-thiol		6-Thioguanine
	2,2,2″-Trichlorotriethylamine (HN3)	Tris(β-chloroethyl)amine		
	Triethylenethiophosphoramide	Tris(1-aziridinyl)phosphine sulfide	Thio-tepa®	
	Triethylmelamine (TEM)	2,4,6-Tris(1-aziridinyl)-s-triazine		
	Vinblastine (VLB)		Velban®	Vincaleukoblastine
	Vincristine (VCR)		Oncovin®	Leuocristine
III (active when given before or after immune stimulus)	Cyclophosphamide	2-[Bis(2-chloroethyl)aminotetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide]	Cytoxan®	Endoxan†
	Procarbazine		Matulane®	Methylhydrazine

* In addition to the drugs listed here, X-irradiation functions as a class I agent.

† Endoxan is a common name in the U.S., an official or generic name in Great Britain.

TABLE 3

Some immunosuppressive drugs

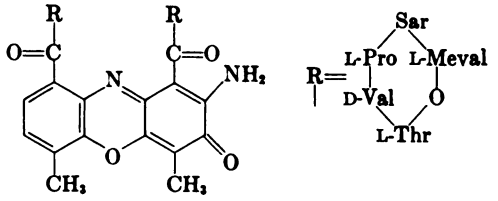
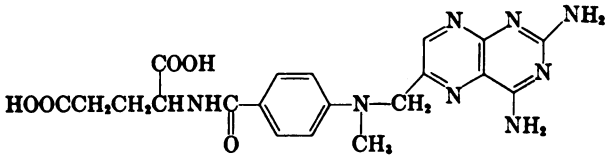
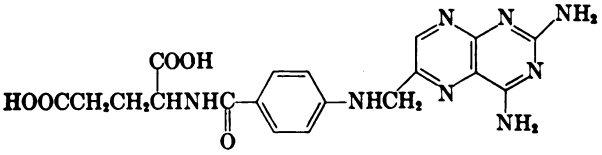
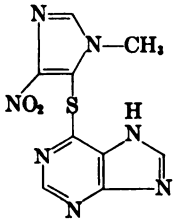
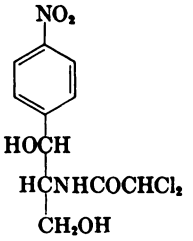
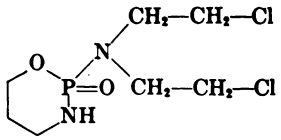
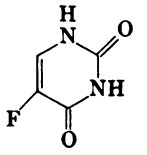
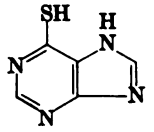
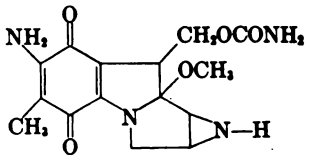
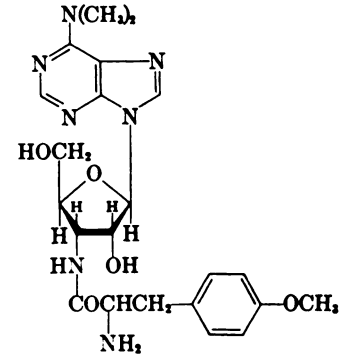
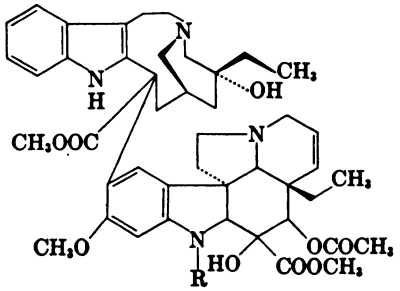
Agent	Formula
Actinomycin D	
Amethopterin	
Aminopterin	
Azaserine	$N_2CHCOOCH_2CH(NH_2)COOH$
Azathioprine	
Chloramphenicol	
Cyclophosphamide	
5-Fluorouracil	

TABLE 3—*continued*

Agent	Formula
6-Mercaptopurine	
Mitomycin C	
Myleran	$CH_3—SO_2O(CH_2)_4OSO_2CH_3$
Puromycin	
Vinblastine (R = —CH_3)	
Vincristine (R = —CHO)	

In a comprehensive study on the effect of several chemical agents on the primary agglutinin response in mice (433), drugs were injected intraperitoneally once daily for 5 consecutive days at doses equivalent to 50% of the LD50. Sheep red blood cells were injected into the animals at various times in relation to the first day of drug treatment, the mice were bled at various intervals from 4 to 28 days, and then the overall mean titer was tabulated. L- and D-phenylalanine mustard lowered the mean titer only when given 2 days before initiation of the immune response. In other studies single doses (50% of the LD50) of L-phenylalanine mustard depressed day-7 agglutinin titers in mice when given before sheep red blood cells, but in rats the compound was not active if given as a

single LD50 dose before or after an intravenous injection of the antigen (360). In man, a single dose of the drug (2 mg/kg) given intravenously 4 hr before immunization with the polysaccharide antigen Vi completely blocked an antibody response in only 1 of 3 persons (358).

L-Phenylalanine mustard was given in 5 consecutive daily doses to mice just before the engraftment of an allogeneic tumor (normally rejected). All mice supported the growth of the tumor. Furthermore, 11 of 16 such treated animals succumbed to progressively growing allogeneic tumors (204). L-Phenylalanine mustard was considered to have the highest therapeutic ratio of several compounds tested for suppressing the homograft reaction to an allogeneic tumor.

This alkylating agent is unusual compared with the other alkylating agents since it is the only such drug of this general group that is clearly a class I agent. Comparative studies of this drug and X-ray on macrophage function, antigen clearance, and distribution of antigen might prove most interesting. Needless to say, comparisons of these agents with other alkylating agents that are more clearly class II would also be pertinent.

The alkylating agent busulfan is noted for its predominant effect on cells of the myeloid series with relative sparing of cytotoxic effects on lymphocytes (119). This compound may function by reacting with sulfhydryl groups of cysteine-containing enzymes and proteins (338). It has been useful therapeutically primarily in the treatment of chronic myelogenous leukemia (93). Busulphan was reported to inhibit antibody formation in mice only if given before and not if given after immunization with a bacterial or heterologous red cell antigen (32, 40). In rats given a single dose of busulfan (60% of LD50) 48 hr before or 48 hr after an intravenous injection of sheep red blood cells, the drug-treated groups showed significantly higher mean agglutinin titers at days 4, 7, 10, 14, 21, 28, and 35 postimmunization when compared to saline-injected controls (356). Also, rats given a lethal dose of busulfan could be protected from dying by the injection of syngeneic (same inbred strain, in which ordinarily there is no genetic or immunologic barrier to transplantation) marrow, but not allogeneic (different inbred strain, in which there are genetically determined immunologic barriers to transplantation) marrow, after the drug. The evidence that busulfan is a class I immunosuppressive agent in the mouse is based on two reports from the same workers. In view of the experience in the rat and the evidence cited above for a relative sparing action of busulfan on lymphocytes, the work with this drug in mice needs confirmation. The increased antibody response reported in the rat also deserves further study.

In a study of the effect of single doses of a number of alkylating agents given to mice at various times in relation to the administration of a bacterial vaccine, mechlorethamine, triethylene melamine, chlorambucil, and thio-tepa were not suppressive if given before the antigen, but were immunosuppressive if given after the stimulus (30). Uracil mustard depressed the antibody response of mice to human globulin only when it was given after the stimulus, but there was no immune suppression in rats given uracil mustard before or after an injection of sheep red blood cells (74). In rats given 5 equal, consecutive daily injec-

tions of mechlorethamine at the dose level of 50% of the LD50, sheep red blood cells were injected 48 hr before the first drug dose, on the day of the first drug dose, or 2 days after the first drug dose (362). No statistically significant suppression was noted either in mean peak agglutinin titer or in induction time. The longest induction times and lowest mean peak titers, however, were seen when the drug was started 48 hr after antigen injection. In a similar study in mice mechlorethamine and chlorambucil were inactive (433). Taliaferro and Taliaferro (412) made the interesting observation that immunity of chickens to malaria (as measured by the severity of the induced disease) was depressed markedly when tris(β-chlorethyl)amine was given after, as compared to before, the injection with *Plasmodium gallenaceum*.

In man mechlorethamine (0.8 mg/kg) given as a single dose 4 hr before immunization with Vi or *Pasteurella tularensis* antigen was not immunosuppressive (369), but when mechlorethamine was given in a dose of 0.1 mg/kg each day for 7 consecutive days and immunization with Vi was performed on the first day of drug treatment, total inhibition of antibody production was seen in 3 out of 7 persons (357, 360). The toxicities of the single dose and of the multiple dose schedule were similar in terms of white count and platelet depression. Existing levels of isoagglutinin titers and established delayed hypersensitivity were not affected by large single doses (0.8 mg/kg) of mechlorethamine (369).

Levinson and Necheles (247) reported that mechlorethamine-treated Holtzman rats exhibited long-term survival of skin allografts. McQuarrie *et al.* (281), however, were unable to confirm these results. They pointed out that Holtzman rats were partially inbred and that allografts often survived for prolonged periods without immunosuppression. In another study lymphohematopoetic grafts could not be established in mice previously given mechlorethamine in the lethal range (323).

In general the available evidence for a number of alkylating agents mentioned in the three preceeding paragraphs suggests that when they have been demonstrated to be immunosuppressive in a given experimental system they have behaved as class II agents. It is clear, however, that more information is needed to validate this impression. Further comparative studies of selected agents in this class with class I and class III alkylating agents in terms of effects of immune processes, distribution in the body, and metabolic transformation would be most interesting and might lead to some explanation of the basis of these varying effects of alkylating agents as a group upon immune processes.

2. Adrenal steroids. Adrenal steroids have become a "staple" as immunosuppressive therapy in a variety of clinical situations, and their effect on defined immune responses has been recently extensively reviewed (154, 270); discussion here will be limited to a few selected references.

Perhaps the most precise work on the effects of adrenal steroids as immunosuppressants *in vivo* is that of Berglund (46, 47) and Borum and Berglund (62). These authors studied the response of mice to sheep red blood cells. The maximal effect of prednisone on the immune response was noted in mice whose treatment with the steroid was begun 14 and 8 hr before antigen, but some suppression

was noted in animals treated as early as 18 hr before or as late as the time of antigen injection. Berglund concluded that the immunological tissue of the mouse must be damaged before the 13th hr after antigen stimulation to influence the response. Further administration of steroids apparently does not need to be sustained. Apparently, all that is needed is an hour of damage at a crucial time in the evolution of the antibody response. These observations were extended to rats, in which similar results were noted (47). Dukor and Dietrich (110) noted maximal suppression of antibody formation when cortisone acetate was given just before foreign red cells were injected into mice. Increasing immunosuppression with decreasing doses of antigen and with the "weaker" antigens was also noted. They stated further that the steroid had a marked effect on the clearance of chromium-labeled foreign red cells and suggested that the immunosuppressive effect of steroids might be accounted for by specific interference in antigen processing.

In view of the success of corticosteroids in the treatment of "immunological diseases" in man, it is of interest, as emphasized by Schwartz (376), that there is no convincing evidence for suppression of antibody synthesis in man. The doses of steroids used in clinical studies of antibody synthesis were quite low as compared with present-day therapeutic dose levels.

The immune response that results in delayed hypersensitivity manifests itself as an immunological specific inflammatory reaction. It is difficult, therefore, to divorce the immunosuppressive effects of adrenal steroids on this reaction from the well-known anti-inflammatory effects of these agents. Tuberculin reactions (a form of delayed hypersensitivity) have been suppressed or modified by cortisone in laboratory animals (102, 113, 156, 319, 404) and in man (438). The rapid recovery from cortisone-induced anergy (250) and the inhibition of tuberculin hypersensitivity by the local application of cortisone (438), however, suggest that we are dealing primarily with a peripheral anti-inflammatory effect.

Billingham *et al.* (52) and Morgan (302) were the first to demonstrate that cortisone can prolong the survival of skin allografts in rabbits. These results were confirmed (234, 452, 453). Application of the compound to the graft itself prolonged the survival (53, 453). In addition, cortisone prolonged the survival of skin grafts in mice (282) and guinea pigs (395, 396). The compound, however, was ineffective in pigs (444), dogs (234), monkeys (235), and man (28, 118, 279, 444). An increase in survival of kidney allografts occurred in dogs treated with 40 mg of prednisone daily (459), and prednisone therapy in doses of 50 to 200 mg daily tended to reverse the acute rejection process in canine renal allografts in animals on azathioprine therapy (274).

Although the adrenal steroids may be classed as immunosuppressive agents, their mechanisms of action in various types of immune response are poorly understood. These agents have effects on phagocytosis (190, 312), lympholytic effects (106, 448), and anti-inflammatory effects (162), as well as a general inhibitory action upon protein synthesis (454). All of these effects may be important in explaining the immunosuppressive effects; it is hoped that they will be sorted out in the future.

3. Antibiotics. Mitomycin C, actinomycin D, chloramphenicol and puromycin are immunosuppressive. Mitomycin C is clearly in class I and actinomycin D in class II, but classification of chloramphenicol and puromycin is not possible at the moment. For sake of clarity all four antibiotics will be discussed in this section.

Mitomycin C, an antibiotic isolated from *Streptomyces caespitosus* (186, 439), has been used as a cancerocidal agent (406). It inhibits DNA synthesis (208, 335, 384), depolymerizing the nucleic acid and thus inhibiting its replication (1). The effect on DNA is seen at low concentrations while RNA and protein synthesis continues; at higher concentrations, RNA synthesis and protein synthesis are also affected (382). Mitomycin C has also been shown to act as an alkylating agent (439).

Mitomycin C inhibited antibody formation to human serum albumin and prolonged survival of skin allografts in mice only when given before the antigenic stimulus, but there was no effect on the serologic response to injected polio virus vaccine (14). If Lewis rats were given mitomycin C on the day of antigen (WF strain lymphocytes) injection or within 24 hr before antigen injection the cytotoxic antibody response (against WF strain lymphocytes) was significantly depressed, whereas mitomycin C given after injection of the antigen was without effect (350). Mitomycin C given after antigen administration did not inhibit the immune response in 4-day-old rabbit recipients of adult rabbit lymphoid cells mixed with *Brucella* antigen *in vitro*, and in 10-day-old rabbit recipients of sheep red blood cells (211).

When mitomycin C was used in an effort to modify the rejection of renal allografts in dogs, the mean survival was almost doubled but toxicity, including serious bleeding, was a major problem (321). In this study the drug was administered before and continued after renal grafting. Mitomycin C could be used to inhibit the capacity of cells from picryl-chloride-sensitized guinea pigs to transfer this form of delayed hypersensitivity to nonsensitized guinea pigs when given at the time of cell transfer (57, 58). Mitomycin C inhibited the graft-*versus*-host reaction induced in newborn F_1 mice by parental spleen cells when the cells were incubated with the drug before transfer (291). The presence or absence of chimerism was not tested for; in fact, the transferred cells may have been killed outright, indiscriminately, by the drug. Further studies of the effect of this antibiotic on immune processes would be informative since much of its biochemistry is known.

Actinomycin D binds the guanine residue of DNA and thereby inhibits DNA-directed RNA synthesis by making the DNA inaccessible to RNA polymerase (165). High concentrations, however, apparently do interfere with DNA synthesis more directly (165). There has been a curious tendency, particularly with this drug, to use the drug as a reagent with only one presumed mechanism of action in order to explain its effects on the immune response. Furthermore, in many instances, rather simple pharmacological principles regarding vehicles of drug administration, *etc.*, have been ignored.

Actinomycin D injected into rats a few hours before the injection of sheep

red blood cells or simultaneously with antigen gave results no different from that of the control, but administration 1 or 2 days after the antigen depressed antibody synthesis with a prolonged lowering of titers (85, 360). Actinomycin C, a related compound, was found to markedly depress 9-day anti-sheep red blood cell agglutinin titers in mice when the drug was given 1 to 4 days after the administration of antigen (68). When this drug was given 72 hr before the injection of sheep red blood cells the 9-day agglutinin titer was higher than that of the control. Other studies in rodents (76, 176, 308, 456) and in chickens (318) have confirmed the immunosuppressive properties of actinomycin D.

Spleens of mice were examined at various intervals after immunization with sheep red blood cells and treatment with actinomycin D, and the histological findings correlated with the agglutinin response (179, 457). It was concluded that damage to the large pyroninophilic cells of the germinal centers was the major cellular site of action of this immunosuppressive drug.

Actinomycin D does not appear to have a high therapeutic ratio for immunosuppression *in vivo* when compared to several other drugs. Nevertheless, it has been of great interest primarily because of its presumed mechanism of action. A number of interesting reports of its action *in vitro* have appeared. Fishman (131, 132) developed a system of antibody production in isolated tissues of rats to T2 bacteriophage. Macrophages harvested from a peritoneal exudate of rats are incubated in culture with antigen and then disrupted; after the material is transferred to cultures of rat lymph node cells, antibody is produced. When antinomycin D was added to the macrophage culture together with the antigen, the early 19 S antibody production was decreased, but the ensuing 7 S antibody production was not (133). The authors suggested that there were in this model system two types of information, only one of which was affected by actinomycin D.

The transformation of the small lymphocytes of human peripheral blood that ordinarily occurs under the stimulus of phytohemagglutinin or specific antigen was blocked by the addition of actinomycin D to the cultures (195).

The effect of actinomycin D on secondary responses *in vitro* has been documented by several groups of investigators (240, 295, 390, 429, 431), and all studies point to an action on RNA metabolism. In delayed hypersensitivity measured *in vitro* the effect of actinomycin D was minimal even at doses that were toxic to cells (96).

The antibiotic chloramphenicol inhibits the binding of messenger RNA to ribosomes (443). This agent, which is widely used for clinical infections, was implicated in causing hematopoetic depression in patients undergoing prolonged treatments (64). Chloramphenicol can inhibit protein synthesis in mammalian cell-free systems as effectively as in analogous microbial systems when mammalian protein synthesis is stimulated by the addition of template RNA (440, 442). It was suggested that chloramphenicol acted by blocking the attachment of messenger RNA to its ribosomal binding site. A similar explanation was offered in another study, in which the ability of chloramphenicol to inhibit antibody synthesis in tissue culture was demonstrated (11). The ability of chlor-

amphenicol to inhibit antibody formation *in vitro* was confirmed *in vivo* in rabbits (441). In the same study the survival of skin allografts was prolonged. For maximal effect chloramphenicol has to be administered within hours of immunization and continued for several days (77, 91). Recently, the suppression of an anamestic response to tetanus toxoid was demonstrated in man (94). The drug was used one day before immunization and continued for 10 to 14 days. Unfortunately it is not possible to place chloramphenicol in a class since in all the above studies chloramphenicol was present (tissue culture) or administered both before and after antigenic stimulation. Further work defining its functional classification would be of interest.

Puromycin, an antibiotic (331), inhibits the amino acid transfer from soluble RNA to ribosomal protein (458). It has been suggested that puromycin inhibits protein synthesis by competing with aminoacyl-tRNA for the growing peptide chains. Once puromycin has combined with these chains the release of the complex from the ribosomes results in incomplete protein chains (310). Many aspects of the activity of puromycin in a variety of systems have been reviewed elsewhere (95, 309).

Puromycin suppressed the antibody response to polio virus in mice when given before or after the antigenic stimulus and suppressed the antibody response to human serum albumin only when given before immunization (14). In the same study skin allograft survival was not affected by administration of drug before or after grafting. It inhibits ongoing antibody production *in vitro* (11, 207, 309). More studies with this compound are needed before it can be placed in a functional class. Such studies would be of particular interest because of its known biochemical actions.

4. Other compounds. Evidence will be presented in this section that phytohemagglutinin is a class I immunosuppressive agent. Although the available evidence does not permit a functional classification for colchicine, it will be discussed here also because of its known effects on phagocytosis, a process important in very early stages of the immune response.

The blastogenic (blast cell formation) and mitogenic effects of phytohemagglutinin on human and animal lymphocytes have been noted by several investigators under a variety of culture conditions (88, 126, 155, 196, 205, 253, 254, 313, 315). The cellular changes are preceded by the synthesis of RNA and DNA (20, 22, 89, 90, 276, 421). These striking events have led various workers to investigate the effect of this substance on the immune response.

In general, the administration of phytohemagglutinin just before an antigenic stimulus depresses antibody synthesis. This holds true for mice and rats immunized with heterologous erythrocytes (120, 217, 397), bacterial antigens (121, 158, 217, 348), or protein antigens (155). Administration of phytohemagglutinin to rats after immunization with chicken erythrocytes enhanced the hemolytic response (120). Two reports would appear to be discordant with the data cited above. In one study an enhanced hemagglutination response was seen when phytohemagglutinin was injected into mice 3 to 5 days before immunization with rat erythrocytes (155). In the other study the rabbit's response

to protein antigens or sheep red blood cells was enhanced (higher antibody titers) by phytohemagglutinin whether it was administered before or after immunization (368). In other studies in which phytohemagglutinin was continuously administered to individual animals both before and after the injection of sheep red blood cells, suppression of the antibody response was seen in rabbits (275), mice, and rats (215, 275). These latter studies attest to the immunosuppressive properties of phytohemagglutinin but provide no discriminating data as to the functional classification of phytohemagglutinin.

The survival of skin allografts was prolonged in mice (349) and rabbits (275) by pretreatment as well as combined pre- and post-treatment (275, 301). In one study, however, combined pre- and post-treatment failed to prolong skin allografts in mice (225). The survival of renal allografts in dogs was prolonged when individual dogs were given phytohemagglutinin before as well as after grafting (78). Combined pre- and post-treatment with phytohemagglutinin did not affect delayed hypersensitivity in mice and rats (214).

Allergic encephalomyelitis is induced in guinea pigs by the injection of homogenized spinal cord suspended in Freund's adjuvant. The expression of the disease is a complex interaction of cellular (delayed hypersensitivity) and humoral (antibody) factors. In one study pretreatment with phytohemagglutinin enhanced and post-treatment suppressed the overt signs of the disease in guinea pigs (385).

Phytohemagglutinin is clearly immunosuppressive in a variety of systems and the majority of discriminating studies support the suggestion that it is a class I immunosuppressive agent.

Colchicine is a plant alkaloid, known primarily in the treatment of gout and experimentally as an inhibitor of mitosis in metaphase. Colchicine-treated phagocytes ingest bacteria, but the granulation, vacuolization, and changes in acid phosphatase activity that normally accompany phagocytic digestion are inhibited (268, 269). Furthermore, colchicine inhibits the increased oxygen consumption that normally accompanies phagocytosis *in vitro*. It would seem that there are at least two possible mechanisms for the immunosuppressive action of colchicine; its inhibition of certain aspects of phagocytic function and its effect as a mitotic poison, which is particularly directed at lymphoblasts.

Colchicine inhibited serum sickness in rabbits when the drug was begun on the day before administration of horse serum (139). Out of 16 surviving animals 11 had less severe arterial and cardiac lesions than the controls. Levels of antibody in response to the horse serum were also depressed. Colchicine also significantly suppressed the antibody response in mice given sheep red blood cells (270) and in rats given bacterial antigen (123). On the other hand in hamsters given sheep red blood cells, colchicine increased antibody titers at all but the largest doses (194). (The paradoxical enhancing effect of immunosuppressive drugs will be discussed in section VI.) At the highest doses used, however, about half of the animals produced no detectable antibody. In a study of the effect of colchicine on established tuberculin delayed hypersensitivity and skin allograft survival no significant immunosuppression was found (135, 136).

Although colchicine does not appear to have a high therapeutic ratio for immunosuppression, further use of this agent in sensitive systems designed to test its functional classification would be of interest because of its effects on phagocytic mechanisms.

D. Class II agents

The majority of immunosuppressive drugs in current use are class II agents and are most effective as suppressants when given a few days after the antigenic stimulus. The period of maximal sensitivity to these agents may be brief, *i.e.*, a day or two after the antigenic stimulus, and may be limited to just one phase of the immune response. In general, proliferation and differentiation of immunocompetent cells seem to be more sensitive to class II agents than other stages. The major action of most of the drugs appears to be killing of cells. Most of the class II agents are ineffective if given only before the antigen, and some of them may actually enhance the immune response if used solely in this way. In addition, they may be ineffective if applied too long after the antigenic stimulus. The relation of alkylating agents to class II was discussed in section IV C 1 and the class II antibiotic, actinomycin D, was discussed in section IV C 3.

1. Purine analogues. The purine analogue azathioprine is the most widely used immunosuppressive drug in clinical organ transplantation, and its use as such can be related historically to the early observations of Schwartz and his colleagues with 6-mercaptopurine (380, 381). Azathioprine was synthesized by Hitchings and Elion (198) in an attempt to increase the therapeutic ratio of 6-mercaptopurine, since azathioprine is converted into this drug by sulfhydryl groups *in vivo* (115).

Schwartz *et al.* (381) noted that when rabbits immunized with bovine serum albumin were treated with 6-mercaptopurine daily for 2 weeks, beginning on the day of antigen injection, profound immunosuppression was seen. Although the primary response to bovine serum albumin was readily suppressed by 6-mercaptopurine, the secondary response to this antigen was unaffected (380). Schwartz and his coworkers have continued their studies of this agent; many of their results are summarized in recent reviews (373, 376). The immunosuppressive effects of 6-mercaptopurine on antibody formation have been amply confirmed in several species with many different antigens, and studies have been extended to include other purine analogues, especially 6-thioguanine and azathioprine in chickens (318), mice (148, 149, 152, 180, 308, 433), rats (363), rabbits (380, 381, 402), monkeys (210), dogs (272), and man (191, 192, 246, 258, 357, 369, 409, 435). The drugs have been irregularly "active" in the guinea pig, however (160, 203, 259, 260).

6-Mercaptopurine has pronounced effects on inflammation (193, 324, 325), and this must be taken into account when considering its effects on delayed hypersensitivity. The delayed hypersensitivity in the rabbit to bovine serum albumin was suppressed by 4 days of drug treatment, but this treatment had no measurable effect on antibody synthesis (61). Similar results were found in rabbits given antigen-antibody precipitates in Freund's adjuvant (316). Borel

(60) was able to show that suppression of delayed hypersensitivity by 6-mercaptopurine was due to its effect on the immunocompetent cells: he could block the development of delayed hypersensitivity to the test sensitizing antigen while animals were responding to antigens to which they had been previously sensitized. An anti-inflammatory agent should have blocked both responses.

Skin graft survival has been prolonged with purine analogues in rabbits (284, 285, 378), goldfish (167, 248), dogs (230, 407), and man (246). Inconsistent results, however, have been reported in mice (202, 280, 284, 403a, 407) and rats (202, 363, 418).

In general, the purine analogues are most effective in suppressing antibody formation (362) or the skin homograft response (363) when given a few days after the antigenic stimulus. It seems most likely that proliferation and differentiation are more sensitive to the action of these agents than other stages of the immune response.

2. Pyrimidine analogues. The analogues of pyrimidine bases have not been studied *in vivo* for their immunosuppressive effects as extensively as other compounds. 5-Fluoro-2′-deoxyuridine and 5-fluorouracil were immunosuppressive in mice; inhibition was most pronounced when the drugs were given 24 to 48 hr after immunization with bovine *gamma* globulin (289). Similar immunosuppression and timing relationships were found with bacterial antigens in the mouse (31). Uy *et al.* (433), by using sheep red blood cells in the mouse, found these drugs inactive as immunosuppressants. On the other hand, 5-bromodeoxyuridine was reported to be immunosuppressive in mice (49). 5-Iododeoxyuridine and 5-bromodeoxyuridine can inhibit antibody formation *in vitro*, and the effects are partially reversed by thymidine (112). Similar results on reversals were noted with 5-fluorouracil (231) and 5-fluoro-2′-deoxyuridine (317). These compounds were immunosuppressive in man given Vi antigen on the first day of a 7-day schedule of the drug, but established delayed hypersensitivity and isoagglutinin titers were not affected (357, 369). Blomgren *et al.* (56) studied the ability of cancer patients to express delayed hypersensitivity to a variety of antigens both before and after therapy with 5-fluorouracil and 5-fluoro-2′-deoxyuridine. Of 41 patients, 20 developed one or more positive skin tests after drug treatment. They suggested that this enhancing effect on delayed hypersensitivity might be related to the anticancer properties of the drug.

Cytosine arabinoside is a new pyrimidine analogue that has "found" clinical use as an antileukemic agent (73). It inhibits hemolysin formation in mice (122, 129, 130, 170), rats (296), hamsters (130), and rabbits (221). It inhibited responses to bovine *gamma* globulin in mice only when it was given after the antigen (74). The primary antibody response was inhibited the most when cytosine arabinoside was given on days 1 to 4 after antigen injection (129). By the hemolytic plaque assay (a technique in which the number of antibody-secreting cells is counted directly after plating in agar) suppression of responses to sheep red blood cells in mice occurred when the drug was given 2 days after the antigenic challenge, the time when the number of antibody-synthesizing cells was increasing logarithmically (170). Multiple doses had a greater immunosuppressive

effect than single doses. Pretreatment with the drug gave no effect. This agent did not prolong the survival of canine renal allografts (10, 171). Cytosine arabinoside also inhibited the development of delayed hypersensitivity in rabbits (220, 221) and experimental allergic encephalomyelitis in rats (220).

Cytosine arabinoside was found to block antibody production to Vi antigen in man (297, 357) as well as prevent the induction of delayed hypersensitivity to 2,4-dinitrochlorobenzene (297). There was no effect on established hypersensitivity (297).

3. Folic acid antagonists. The potential of folic acid antagonists as immunosuppressants was suggested by Little (249), who noted lower antibody titers to *Brucella abortus*, *Salmonella typhosa*, and *Pasteurella multocida* while feeding chickens a diet deficient in pteroylglutamic acid from the time of hatching. Of particular interest was the finding that 4-aminopteroylaspartic acid (amino-anfol) perpetuated the immunological defect when given at the same time as pteroylglutamic acid, in essence a demonstration of immunological suppression by a folic acid antagonist.

The two most common members of this class, aminopterin and methotrexate, are well characterized as to their mode of action in immunosuppression (45). They inhibit the enzyme dihydrofolate reductase, thus preventing the conversion of folic acid to tetrahydrofolic acid. This step is necessary for the synthesis of many biochemical compounds, including DNA, RNA, and several coenzyme species.

Suppression of hemolysin titers to sheep red blood cells was dose-related in mice given methotrexate in five equal doses, with treatment beginning on the day of antigen administration (270). The greatest block of antibody synthesis to typhoid-paratyphoid A and B vaccine in the mouse by a single dose of methotrexate occurred when it was given 2 days after the antigen (31). The same was true with sheep red blood cells in mice (433). In rats given five daily doses of methotrexate at 50% of the LD50 (maximal tolerated doses) started at various times in relation to the day of antigen injection, the drug had the greatest effect when begun 1 to 2 days after antigen injection, but was completely ineffective when antigen was given a day or two after completion of a 5-day course of drug (362). In addition, there was no effect of the drug upon serum antibody titers when administration of the drug was begun 5 days after the antigen.

Methotrexate completely suppressed antibody formation to either diphtheria toxoid or ovalbumin in guinea pigs (146 147). High doses of the drug were required when antigens were emulsified in complete Freund's adjuvant. Only partial suppression of circulating antibody synthesis occurred, however, in guinea pigs treated with methotrexate and challenged with human serum albumin in incomplete Freund's adjuvant (233). Formation of antibody to ovalbumin in guinea pigs could be suppressed by administering methotrexate up to the time when small amounts of antibody appeared but not when production was at its height (63). The secondary response was suppressed when daily administration of the drug began at the time of administration of antigen or 48 hr later. Methotrexate could suppress antibody synthesis in dogs (419), but not

rabbits (66) but, aminopterin could completely suppress the immune response in 3- to 5-day-old rabbits receiving adult rabbit spleen cells mixed with *Brucella suis* antigen *in vitro* (403).

Methotrexate has a high therapeutic ratio for immunosuppression in man given Vi antigen (192, 357, 369). Antibody titers were suppressed to a greater degree when antigen was given at the beginning of drug therapy than when given later (192). Methotrexate suppressed antibody production in man to the hemocyanin derived from the keyhole limpet and to diphtheria toxoid (409).

The administration of folinic acid to leukemic mice 12 or 24 hr after the antagonist aminopterin resulted in a greater therapeutic effect of aminopterin (166). The same was true with methotrexate (282). Mice given folinic acid 8 hr after a single large dose of methotrexate gained considerable protection against death and weight loss, yet their immune response to typhoid-parthyphoid A and B vaccine was profoundly inhibited (42). By appropriately spacing the rescue doses of folinic acid the investigators demonstrated that methotrexate required only 6 to 8 hr to complete its action on the immune system.

Mitchell *et al.* (297) used this principle in man. Primary antibody response to Vi antigen and secondary antibody response to tetanus toxoid were measured. The antigens were injected 15 to 30 min before initiating methotrexate infusion followed by folinic acid infusion. Infusion of methotrexate followed by folinic acid was performed repeatedly (up to 11 times) in 20 patients. Overall toxicity was relatively slight, with a mean nadir of leukopenia of 3600 cells per mm^3. Complete suppression of antibody response to Vi and tetanus toxoid was noted as long as the methotrexate-folinic acid infusion was continued. Furthermore, 9 of 20 patients failed to develop a primary response, even after cessation of therapy. Regardless of the number of infusions given, antibody appeared in the serum of 11 patients approximately 12 days after the last infusion.

Aminopterin inhibited both established and newly acquired tuberculin hypersensitivity in guinea pigs (145, 333). Friedman *et al.* (146) later found that methotrexate depressed delayed hypersensitivity to diphtheria toxoid and ovalbumin in the guinea pig. In contrast to the finding of Borel and Schwartz with 6-mercaptopurine in rabbits (61), methotrexate inhibited the primary antibody response at a lower dose than that required for suppression of delayed hypersensitivity. In subsequent experiments, it was found that tuberculin reactions suppressed by methotrexate became positive 10 days after cessation of drug (145). Several studies in man have failed to show an effect of methotrexate on established delayed hypersensitivity (192, 297, 369). Mitchell *et al.* (297), however, demonstrated that it could block induction of delayed hypersensitivity to dinitrochlorobenzene.

Turk and Stone (426) studied the dynamics of the large pyroninophilic cells and small lymphocytes involved in the hypersensitivity response by autoradiography and examination of imprints as well as of sections taken from regional lymph nodes during sensitization. Methotrexate did not block the formation of the large pyroninophilic cells that were normally seen in response to sensitization, but acted primarily by inhibiting the development of a population of small lymphocytes that appeared to be derived from the large pyroninophilic cells.

Methotrexate has been reported to prolong skin grafts in mice (164, 407), rats (363), guinea pigs (33), dogs (187), and fish (248), but failure to prolong skin grafts has been reported in rabbits (66, 284). It prolonged the survival of allogeneic skin grafts in rats (363). In this study, the optimal time of administration of methotrexate was 5 to 7 days after placing of the skin graft. A marked prolongation of skin graft survival in guinea pigs, with reduced overall toxicity occurred when methotrexate injections were followed by appropriately spaced injections of folinic acid (35).

Methotrexate has a high therapeutic ratio for immunosuppression in a variety of species. Together with the purine analogues and cyclophosphamide (*vide infra*), this drug has become a powerful laboratory tool as well as an important addition to clinical immunosuppression. Further studies with this agent will be awaited with interest.

4. Vinca alkaloids. Both vinblastine and vincristine are mitotic spindle inhibitors like colchicine and arrest mitosis in metaphase (327, 411, 432); they have found clinical use primarily in the treatment of lymphomas, Hodgkin's disease, and the leukemias. Immunological suppression with these compounds has not been impressive. Antibody formation in rabbits was not affected by these compounds (144). Vinblastine prolonged the induction time of antibody synthesis in rats when given 2 days after the injection of sheep red blood cells. Vinblastine given before or simultaneously with antigen did not result in immune suppression (262). Both compounds were inactive in mice given sheep red blood cells (433). On the other hand, vinblastine suppressed the antibody response in mice if given 2 days after typhoid-paratyphoid A and B vaccine (31). Furthermore, both drugs inhibited both antibody production and delayed hypersensitivity to bovine serum albumin in rats (5, 8). The survival of skin grafts was also prolonged at toxic levels of the drugs (5, 8).

Although these drugs are not impressive as immunosuppressive agents, they have been used very effectively for cytokinetic studies of immune response. When vinblastine was given to mice just after injection of sheep red blood cells the number of antibody-synthesizing cells (by the hemolytic plaque assay) was reduced, but there was no reduction if it was given before the sheep cells (411). Vinblastine was inactive when given before 12 hr, but its immunosuppressive effect was maximal when given at 15 hr after the antigen (327). Shortly thereafter, the population of antibody-synthesizing cells began to rise exponentially over the background level. These results indicate that the precursor cells are normally in a resting state and not in cell cycle. When they are stimulated by the antigen, directly or indirectly, they enter the G_1 phase of the cell cycle, then into the S (DNA synthesis) phase, the G_2 phase, and finally the M (mitosis) phase which completes the cell cycle. After mitosis they change into immature antibody-synthesizing cells. It would appear that the cells were in M phase 15 hr after antigen stimulation. Since the mean generation time of antigen-stimulated blasts cells is about 9 hr (344), these results further indicate that the precursor cells went into cell cycle about 6 hr after antigen injection. This suggests that the antigen-processing and the interaction of antigen-reactive cells with

precursor cells, according to the two-cell interaction model (see fig. 1), required 6 hr.

E. Class III agents

These agents may be immunosuppressive if given either before or after the antigenic stimulus and thus share properties with class I and class II agents. Cyclophosphamide is the only agent clearly in this class. Tentatively procarbazine is also placed in this class.

Procarbazine has recently found clinical application in the treatment of Hodgkin's disease and lymphomas (72, 92, 278). A number of enzymes are influenced by this drug and it has been suggested that the observed cytotostatic effects probably reflect alkylation of DNA (445). Other studies with Ehrlich ascites tumor *in vitro* indicated that procarbazine inhibited DNA synthesis (137).

Procarbazine suppressed antibody production in mice to human albumin and polio virus when given before or after the antigen (13). In the same study skin allograft survival was unchanged when drug was given before grafting but prolonged when drug was given after skin grafting. When procarbazine was given to mice daily for one week before skin grafting and continued until rejection occurred, skin graft survival was prolonged (134, 136). Mouse tumor graft survival was prolonged in rats when given procarbazine (59).

Clearly more information is needed regarding the effects of procarbazine on the immune response. Its functional classification is primarily based on one report (13).

The most widely used alkylating agent in immunosuppression is cyclophosphamide. This agent is converted to its active form in the liver (136a). Cyclophosphamide has proved to have one of the highest therapeutic ratios of the many immunosuppressive drugs studied in the rodent. The drug is active if given before or after the antigenic stimulus, but its effect is greatest when given after the antigen (357). Stender *et al.* (400), the first to study this drug, found complete suppression of antibody response to *Brucella* antigen when cyclophosphamide was given before antigen or as late as 4 days after the antigenic stimulus. The drug was able to suppress antibody synthesis even when given after antibody appeared in the serum (401). Similar results were obtained in studies with sheep red blood cells as antigen in the rat (362) and mouse (433). Cyclophosphamide has a higher therapeutic ratio for immunosuppression than does X-ray (358, 366, 368).

In single doses cyclophosphamide suppressed antibody formation in mice if given before or after antigen. The greatest effect, however, was seen when the drug was given a few days after the antigen (31, 32). The maximal sensitivity to the drug occurs between 24 to 48 hr after the injection of sheep red blood cells in mice (150). The proliferative and differentiating events of the immune response are more sensitive to the action of cyclophosphamide than other stages of the immune response (150, 362). Nevertheless, as stressed by Santos and Owens (366) the drug when given before the antigenic stimulus in rodents has a high therapeutic ratio for immunosuppression. Other workers have confirmed

the immunosuppressive effect of cyclophosphamide on antibody production in mice (127, 177), rats (332, 371), and guinea pigs (256, 257, 259).

Santos *et al.* (361) have extensively studied the effect of cyclophosphamide on antibody formation in man. The drug was administered as single or multiple injections at several different dose levels. It was not reproducibly immunosuppressive if given solely before the antigenic stimulus (Vi or *Pasteurella tularensis* vaccine), but had considerable effect when given a day or two after it.

Cyclophosphamide significantly prolongs the survival of allografts in mice (15, 136, 141, 407), rats (363), guinea pigs (36), and rabbits (65, 219). In mice skin allograft survival is prolonged if cyclophosphamide is given before or after grafting. The greatest prolongation of skin allograft survival occurred when it was given a few days after placing of the grafts (41). In rats single or multiple doses of cyclophosphamide given before or after grafting prolonged skin allograft survival. Administration of the drug 5 to 7 days after grafting had the greatest effect (363).

Cyclophosphamide is also markedly inhibitory to tuberculin sensitivity and contact sensitivity in guinea pigs (255, 424–426). When this drug was given to guinea pigs 2 days after sensitization, 9 out of 10 animals failed to react; when the drug was given 4 days after sensitization, 6 out of 10 animals failed to react; but all animals sensitized 16 days before drug treatment had normal responses (424). Positive reactions often return 48 to 72 hr after stopping daily injection of the drug. Turk and Stone (426) concluded on the basis of histologic study that the major effect of cyclophosphamide was to block the appearance of the large pyroninophilic cells that in turn give rise to the "effector" lymphocytes, whereas methotrexate (section IV D 3) did not affect the appearance of these cells but acted primarily by inhibiting the development of a population of small lymphocytes derived from the pyroninophilic cells.

F. Selective effects on 19S and 7S antibody response

As stated earlier (section II D) in a number of animal systems the primary antibody response is made up of two distinct phases. The initial antibody appearing in the serum is a protein of high molecular weight, 19 S. Later an antibody of lower molecular weight, 7 S, appears and, as it rises, the levels of 19 S antibody decline. Although there are several classes of 7 S immunoglobulin (*e.g.*, immunoglobulin A, immunoglobulin G), most of the studies deal primarily with immunoglobulin G antibody.

The immunosuppressive agents that have been tested decrease production of 7 S antibody preferentially and prolong the production of 19 S-antibody. This has been shown with X-ray and 6-mercaptopurine in rabbits (345, 346, 391, 408), with methotrexate in mice (55), with methotrexate and cyclophosphamide in rats (365), and with 6-mercaptopurine, azathioprine, methotrexate, and cytosine arabinoside in man (297, 357, 409). Sahiar and Schwartz (347) have suggested that 19 S and 7 S antibodies were produced by two different cell lines and that the line of cells producing 7 S antibody was inherently more sensitive to the action of the several cytotoxic agents. However, studies with a cell trans-

fer system indicate that 19 S and 7 S antibody-synthesizing cells of mice have identical sensitivities to X-ray (264), methotrexate (359), cyclophosphamide (359), and 6-mercaptopurine (359). It has been suggested that the mechanism that changes an animal's 19 S antibody production to 7 S antibody is the most sensitive phase of the primary immune response. The mechanism for changing the response of an animal from 19 S to 7 S may involve the events that proceed from antigen trapping in the germinal center to 7 S antibody production, which in turn finally turns off 19 S antibody synthesis. Current evidence (3, 178) strongly indicates that the germinal centers, which are very easily damaged by X-ray (212, 311), are concerned with the initiation of 7 S antibody production.

V. DRUG-INDUCED IMMUNOLOGICAL TOLERANCE

One of the more exciting developments in the field of immunosuppression has been the discovery of drug-induced immunological tolerance. In 1959, Schwartz and Dameshek (377) first demonstrated specific drug-induced immunological tolerance. When human serum albumin was injected into rabbits on the first day of a 2-week course of 6-mercaptopurine, no antibody was produced and subsequent challenges with human serum albumin failed to evoke detectable antibodies, although responses to other antigens were quite normal. These findings were soon confirmed (307). The percentage of rabbits made tolerant to bovine serum albumin by the treatment was directly related to the initial dose of antigen. Increasing the dose of 6-mercaptopurine also increased the percentage of tolerant animals (379). LaPlant *et al.* (236) and Forsen and Condie (140) found that even rabbits sensitized to bovine serum albumin could be made tolerant to bovine serum albumin if sufficiently large doses of 6-mercaptopurine were employed. Three of six patients challenged with Vi antigen on the first day of 6-mercaptopurine treatment that was continued for 18 to 24 days failed to respond to that antigen on restimulation 3 to 4 months after the 6-mercaptopurine treatment was discontinued. Their ability to respond to other antigens was unimpaired at that time (246). A single patient given an injection of Vi antigen 24 hr before a 7-day course of 6-mercaptopurine did not respond to Vi antigen but had the usual antibody response to an injection of *Pasteurella tularensis* antigen given after the end of treatment (357).

Methotrexate induces immunological tolerance in at least two systems. Adult mice infected with an otherwise fatal dose of lymphocytic choriomeningitis virus and treated with methotrexate did not die (185). The mice had prolonged viremia, failed to develop meningitis, and resisted reinfection. Precisely the same conditions resulted when this virus was injected into newborn mice without methotrexate. Tolerance to the lymphocytic choriomeningitis virus could be induced by a single dose of methotrexate (200). The maximal yield of tolerant mice was achieved when the methotrexate injection coincided with the peak titer of virus particles in plasma (4 days after inoculation). Rats could be made specifically tolerant to sheep red blood cells if the antigen was injected 2 days before a 5-day course of methotrexate given at a level of 50% of the LD50 (357). These animals could respond to human red blood cells, but were unreactive to

the test antigen. The degree of tolerance or the percentage of animals tolerant was proportional to the dose of sheep red blood cells used in the initial antigen injection. This relationship confirms the importance of the dose of antigen employed to produce tolerance as initially reported by Schwartz and Dameshek (379) in the rabbit by using a protein antigen and 6-mercaptopurine.

Cyclophosphamide has been very successful in the induction of immunological tolerance. Guinea pigs were given cyclophosphamide for 8 days, beginning the day of injection of egg albumin, and 3 months later a second injection of the antigen was given. When challenged by an intracardiac injection of the antigen 3 weeks after the second injection of egg albumin, 73% of the controls died of anaphylactic shock, whereas only 9% of the cyclophosphamide-treated guinea pigs died (256).

Salvin and Smith (354) studied the specificity with which cyclophosphamide can induce immunological tolerance in guinea pigs. The animals were treated with cyclophosphamide and challenged with a hapten-protein conjugate. Two months later, the antigen emulsified in complete Freund's adjuvant was reinjected. Guinea pigs so treated failed to respond with either immediate or delayed hypersensitivity reactions, and serum antibody could not be detected. However, when either the hapten or protein portion of the conjugate was altered, an immune response to the newly substituted portion developed. Nevertheless, tolerance to the original conjugate persisted. They concluded that cyclophosphamide induced tolerance toward the whole antigen molecule. In the guinea pig it is possible to induce tolerance specifically to brain antigen (353) or to thyroid antigen (352). By appropriate scheduling of drug and antigen injection, one can prevent guinea pigs from developing allergic encephalomyelitis or allergic thyroiditis. More important, these animals later cannot be induced to develop allergic encephalomyelitis or thyroiditis despite further attempts at immunization. In mice a single dose of cyclophosphamide can induce tolerance toward heterologous erythrocytes (6, 104, 151). Aisenberg and Davis (7) noted that if thymectomy was performed after initiation of the tolerant state the persistence of tolerance lasted longer than it would if the thymectomy had not been done.

Santos *et al.* (361) demonstrated that specific nonreactivity to an antigen might be induced in man by cyclophosphamide. Patients were given either Vi antigen or *Pasteurella tularensis* antigen a few days before a course of cyclophosphamide (7 mg/kg daily for 7 days). One day after completion of the therapy, the patients were challenged with the original antigen as well as a new antigen. The patients responded normally to the second antigen, but failed to react to the first antigen. Cyclophosphamide could also induce specific tolerance in mice to cells of another histoincompatible strain (367). In this system, the survival and function of spleen cell grafts was measured by the amount of antibody they produced. Recipient mice were given 100 mg/kg of cyclophosphamide. At this dose, they failed to develop antibody after the injection of sheep red blood cells. However, they retained enough immunologic capacity to reject spleen cells from a histoincompatible mouse, and the injection of such spleen cells together

with antigen (sheep red blood cells) produced no antibody. If, however, spleen cells from one strain of mice were injected intravenously 24 hr before administration of cyclophosphamide, the mice would subsequently accept spleen cells from the strain that donated the first injection of spleen cells but not from other strains of mice. To produce this tolerant state optimal conditions were present for both the route of administration and time of administration when donor spleen cells were given intravenously 24 hr before the injection of cyclophosphamide. Subsequently it was demonstrated that one could use this principle to obtain marrow grafts in cyclophosphamide-treated dogs (405). Dogs were given an infusion of blood from a female donor. Twenty-four hours later, they were given 100 mg/kg of cyclophosphamide (LD100). Twenty-four hours after the cyclophosphamide, marrow was transplanted from the donor. This maneuver yielded successful transplants as demonstrated by chromosome analysis in a number of dogs. Encouraged by these reports, Santos *et al.* (361) and Bach *et al.* (19) used this principle of drug-induced immunological tolerance to obtain marrow grafts in man. In both of these studies, donor antigen in the form of peripheral whole blood was injected intravenously 24 hr before a 4-day course of cyclophosphamide. Twenty-four hours after the last dose of cyclophosphamide, the donor marrow cells were injected intravenously. Proof of marrow engraftment was obtained by chromosome analysis. Donor marrow persists in one patient for over a year and persisted until death from various causes in the other patients.

The above studies indicate that immunological tolerance can be induced with several drugs in a variety of species. Furthermore, tolerance is highly specific for the antigen given just before the drug administration or at the beginning of drug administration. The degree of tolerance is influenced by the amount of antigen used, the timing of drug treatment, and the dose of drug employed.

A possible mechanism of the specificity of drug-induced immunological tolerance has been offered by Schwartz and Dameshek (378). According to this view, the first injection of antigen given together with the immunosuppressive drug selects the immunocompetent cells responsive to it and causes them to undergo proliferation and differentiation. These cells are selectively killed because they are more sensitive to the cytotoxic action of the immunosuppressive agents than the unstimulated immunocompetent cells. When the same antigen is given later, there are no cells left that are able to respond to it, but other antigens can arouse an immune response in appropriate surviving cells.

VI. THE ENHANCING EFFECT OF IMMUNOSUPPRESSIVE DRUGS ON IMMUNE RESPONSE

A. Enhancement

Under certain conditions, organisms whose immune system has been partially impaired by immunosuppressive agents respond to an antigen more vigorously than normal organisms. This is what is meant when it is stated that immunosuppressive agents may actually behave as "immunological adjuvants." Gen-

erally an increase in antibody response is observed when antigen is administered shortly before or after the drug or X-ray treatment. This paradoxical phenomenon was first observed by the early radiation immunologists over 50 years ago (224, 273) and since then has been confirmed by others (105, 189, 413, 415). It was recognized that colchicine could enhance antibody synthesis (415, 421), particularly when it was given in a relatively large dose just before the antigenic stimulus (424).

A single injection of 5-fluoro-2′-deoxyuridine enhanced antibody synthesis in mice if given 24 hr before or after challenge with bovine *gamma* globulin; both 19 S and 7 S antibody titers were increased (289, 290). A similar enhancement of antibody production occurred in mice treated with single doses of uracil mustard or cyclophosphamide 1 hr before the injection of bovine *gamma* globulin (74). Rabbits treated with 6-mercaptopurine developed hyperplastic lymphoid tissues 5 to 7 days after a 1-week course of the drug, and antibody formation was enhanced when the bovine *gamma* globulin was injected at the time of maximal lymphoid hyperplasia (80). This enhancing effect was seen as early as 2 or as late as 20 days after the drug was discontinued. Enhancement was most pronounced with low doses of antigen and absent with larger doses. This enhancing or adjuvant effect has also been observed with mechlorethamine (362), busulfan (356), and thioguanine (148). It is of interest that enhancement has been seen in man with methotrexate and azathioprine (409). At least one report suggests that 5-fluorouracil and 5-fluoro-2′-deoxyuridine may enhance delayed hypersensitivity reactions in man (56).

B. Possible underlying cellular mechanism

Insight into this mechanism probably began with the classical studies of Jacobson *et al.* (209) in 1949. Taking advantage of the early lead-shielding study of Chiari (83) and local X-irradiation studies of others (224, 273), Jacobson and his colleagues observed that rabbits given 500 to 800 r to the total body while their spleens were shielded with lead responded almost normally to the test antigen; and 800 r is known to destroy practically all the immunocompetent cells. Thus, after destruction of the majority of the immunocompetent cell population, the surviving minority in the lead-shielded spleen responded to the test antigen so vigorously that the overall response of the X-rayed rabbits was almost normal (209). One implication of these results is that normally, in a maximal antibody response, some unknown restrictive factors allow only a fraction of the total immunocompetent cell population to participate.

Subsequently Taliaferro and Taliaferro (414) performed the reverse experiment; *i.e.*, they performed "radiation splenectomy" by exposing only the exteriorized spleens to X-ray doses as high as 10,000 r while the rabbits were lead-shielded. When the test antigen was administered to these rabbits immediately after the radiation treatment, they responded by generating more antibody than the normal control rabbits. Graham *et al.* (168, 169) also observed an increase in antibody response in rabbits when antigen was injected into the thigh before local irradiation of the injected site with 1000 r. In contrast, the im-

munological response of surgically splenectomized organisms is generally lower than normal (262). Rats whose spleens had been exposed to 10,000 r shortly after administration of antigen had a greater antibody response than unirradiated controls (388); lead-shielded immunocompetent cells were shown to have migrated from elsewhere into the heavily damaged spleen, then rapidly proliferated and differentiated into antibody-synthesizing cells. Furthermore, it was clear that most of the antibodies in the blood of these rats were synthesized by cells that had settled in the spleen.

In contrast to the above "endocloning" (redistribution of endogenous cells) studies of immunocompetent cells, studies on infusion into immunologically inert recipients of dispersed immunocompetent cells from various donor tissues can be called "exocloning" studies (267). As stated earlier in our review, (see II B) this latter model system has generated much of the current data on cellular kinetics of immune response, and the following are some of the key findings that may shed some light to this paradoxical phenomenon of enhancement.

1) There can be as many as 100 times more immunocompetent clones responsive to a test antigen in organisms undergoing a secondary antibody response than in those undergoing a primary response, but this difference may not be detectable, especially when one is using a highly immunogenic test antigen (9, 264, 330).

2) Immunocompetent cells undergoing a secondary response are as radiosensitive as those undergoing a primary response (264), but organisms undergoing a secondary response are more radioresistant than those undergoing a primary response (213, 262, 264, 330). Furthermore, there is a threshold effect to X-ray in both types of organism. For example, in secondary responders the X-ray doses to mice maximally immunized to sheep red blood cells must be greater than 400 r before a significant suppression can be observed, and 400 r has been shown to destroy 95% of the immunocompetent cells (264). This means that the 5% surviving immunocompetent cells after 400 r exposure generated as much antibody as those in unirradiated mice. It would seem then that normally only about 5% of the total immune potential is expressed in a secondary response, assuming there is no significant difference between the functional cells of irradiated and unirradiated mice. This means that in order to demonstrate the effectiveness of an immunosuppressive agent one must be able to destroy more than 95% of the immunocompetent cells of a previously immunized mouse. If, on the other hand, the dose of the drug were below that which would have killed 95% of the immunocompetent cells and were administered in such a way as to create an environment for the expression of more than 5% of the full potential, then an above-normal response would be expected.

3) In reconstitution studies *in vivo* (as described in section II B), involving drug-induced immunologically inert recipients, genetically incompatible spleen cells generated more antibody to sheep red blood cells than genetically compatible spleen cells (366, 368). It is known that in the former case host cells are being destroyed because of graft-*versus*-host reaction at the same time that immunocompetent cells responsive to sheep red blood cells are undergoing pro-

liferation and differentiation. Furthermore, it has been shown repeatedly (*e.g.*, 262) that cell-impermeable diffusion chambers (section II B) containing spleen cells from previously immunized mice and the test antigen, which can be either particulate or soluble, when implanted intraperitoneally into X-rayed recipients can generate 10 times more antibody and antibody-synthesizing cells per unit number of spleen cells than *in situ*. However, this difference is not observed when the chambers are implanted into unirradiated recipients. These results indicate that the superior performance (over those *in situ*) of antigen-stimulated spleen cells in recipients whose lymphoid tissues have been severely damaged is due to more "space" for growth and to the increase in blood- and lymph-borne factors that are essential for proliferation and differentiation.

These endocloning and exocloning studies show that the paradoxical phenomenon of enhancement of immune response by immunosuppressive agents is explicable at the cellular level, provided that two concepts be taken into consideration. 1) The maximal immune response an organism undergoes after administration of an antigen may not necessarily reflect its full immunological potential. 2) The ratio of immunological expression to immunological potential is dependent upon the availability of space for growth and the relative amount of factors essential for proliferation and differentiation. It follows that an immune response can be enhanced most readily by an immunosuppressive agent if the latter can cause enough cell destruction to permit the factors essential for proliferation and differentiation of immunocompetent cells to become plentiful. However, the dose should be low enough that the percentage of immunocompetent cells destroyed is less than the percentage normally expressed in an organism. For example, take the case of immunized mice, in which a maximal secondary response is an expression of about 10% of the immunological potential. Administration of a drug at a dose that will kill *over 90%* of the total population of immunocompetent cells will suppress the immune response. If, however, the drug is administered at an appropriate time at a dose that will kill *only 50%* of the total population of immunocompetent cells and a number of other cells sufficient to increase the factors essential for the surviving immunocompetent cells to proliferate and differentiate maximally, then there could be a response as much as five times that of the normal secondary response.

An alternative to this concept of a balance between immunological expression and immunological potential is the more difficult one which takes into consideration variation in the rate and number of cell division and the rate of differentiation. We purposely did not elaborate on this alternative because, in addition to its complexity, the existing data on this subject and related areas strongly favor the former. Finally, it should be noted that this former concept can also be invoked as a working hypothesis in other systems of immunosuppression, in which contradictory results have been observed, as for example the effect of antigen competition on immune response (4).

VII. CHOICE OF AGENTS FOR CLINICAL USE

The choice of an agent for clinical use will depend in part on the aim. Thus, for the prolongation of renal grafts, and perhaps for the treatment of autoim-

mune diseases, class II or class III agents are considered to be most useful. On the other hand, if one wishes to perform a marrow transplant, the choice of drugs would be limited to class I or class III compounds, unless one wishes to use the principle of drug-induced immunological tolerance as outlined above. Administration of drug after the transplantation in this situation might destroy the transplant itself.

The choice of a particular drug in a given operational class depends upon several factors: the therapeutic ratio for the desired effect as determined in clinical trial; the preference of the patient for one type of toxicity over another (*e.g.*, the gastrointestinal disturbance seen after methotrexate *versus* the neurotoxicity of vinblastine); as well as the metabolism of the drug. Methotrexate, for example, would be a poor choice for prolonging renal homografts simply because most of the drug is normally excreted unchanged in the urine. It would be difficult, therefore, to select a safe dose level in a situation in which the renal function was compromised. This drug might also be dangerous in a patient with nephritis. In general, the more drug one uses, the more profound the immunosuppression. Since titrating the desired effect in the clinical situation is often difficult or impossible, the effort in initial clinical trials should be to use the drug at maximally tolerated doses. Adjustment of the drug dose will depend upon keeping the levels used at a point at which the resulting hematopoietic or other toxicity is recognized, but kept manageable, in much the same way that agents are used as chemotherapeutic agents in patients with cancer.

Apart from the clinical monitoring required in the use of these agents, the clinician should also be thoroughly cognizant of the properties of the particular drug he is employing. Allopurinol, for instance, should not be used with 6-mercaptopurine or azathioprine, since blocking of the enzyme xanthine oxidase by allopurinol will also block the degradation of these drugs and make adjustment of the drug dosage difficult.

It should also be realized that when the drugs are used in the treatment of autoimmune diseases, many of the benefits from an immunosuppressive agent may result from its anti-inflammatory action rather than its immunosuppressive effect. This has recently been emphasized by Swanson and Schwartz (409).

Apart from the immediate acute effect of these agents on the hematopoietic and other systems, long-term use of immunosuppressive agents, as is required in organ grafting, may result in the appearance of certain infections, such as those caused by cytomegalic virus or various fungi and yeasts.

In addition to these hazards, certain alkylating agents have been shown to be carcinogenic in animals. Furthermore, with the advent of renal grafting on a large scale, the occurrence of malignancies has been noted in patients after chronic immunosuppression (326).

Use of immunosuppressive drugs in the clinical setting at present is experimental. These agents can cause acute toxicity and death and have, as well, a potential for far more subtle but equally dangerous long-term effects. As in almost all therapeutic situations, their proposed use requires careful weighing of the expected clinical benefits against the hazards of acute and long-term drug toxicity.

VIII. CONCLUSION

Progress in immunosuppressive drugs during the past decade has been phenomenal. The experimentalists and clinicians have at their disposal a whole battery of drugs, including prednisone, 6-mercaptopurine, azathioprine, methotrexate, and cyclophosphamide. However, newer drugs with higher therapeutic ratios are needed, the mechanisms of action of the outstanding drugs need to be resolved at the cellular and intracellular level, and better methods are needed to restrict the action of drugs to the immunocompetent precursor cells that are responsive only to the test antigen. Fulfillment of these demands will require the concerted efforts of imaginative specialists from several disciplines, including biochemistry, pharmacology, genetics, medicine, and immunology.

The consensus on the cellular mechanism of immune response and induction of immunological tolerance was discussed briefly before the general discussion on the suppressive and enhancing effects of immunosuppressive drugs. The reason for taking this approach was to emphasize that, although the cellular and biochemical events of an immune response are complex, the major pathways are now known (see fig. 1). This might allow the readers to make guesses as to where the sites of action of the various drugs are and to formulate more definitive experiments. It is hoped that future investigators will include, among their techniques, model systems that will enable them to deduce the suppressive and stimulatory effects of drugs and other agents on antigen-reactive immunocompetent cells, immunocompetent precursors of terminal effector cells, immature and mature effector cells, phagocytes, cells engaged in complement formation, and cells involved in the inflammatory events associated with immediate and delayed hypersensitivities. Thus, for example, in dealing with the mechanism of action of drugs on the precursors of terminal effector cells, one would like to know how a drug can affect their receptors for antigen-reactive cells and "processed antigens," their capacity to change from resting to proliferating cells, and their capacity to differentiate into functional effector cells.

At the present rate of progress it is conceivable that within the next decade the problem of immunosuppression may be practically solved to the extent that routine, simple methods may be available to rapidly induce and terminate tolerance to a limited number of antigens without causing serious damage to the immunological and other vital tissues of the recipient.

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APPENDIX

Tabulation of the effects of certain immunosuppressive drugs on the immune responses of various animals

TABLE 1

Effect of nitrogen mustards and alkylating agents on the immune response

Agent	Dose[a]	Animal	Time of Treatment Relative to Initiation of Immune Response			Primary	Secondary	Reported Effect[b]	References
			Before	Same	After				
5-Bis-(2′-chloroethyl) aminouracil	10 μg/day	Mouse			+	+		E–S	222
	1 mg/kg	Rat			+	+		N–E	74
Busulfan	100 mg/kg	Mouse	+			+		M–S	40
	15 mg/kg	Rat	+		+	+		E	356
Chlorambucil	10–30 mg/kg/day	Mouse	+		+	+		N–S	103, 433
Cyclophosphamide[c]	80–350 mg/kg/day	Mouse	+		+	+		S	150, 433
	4 mg	Mouse	+		+	+		S	127
	330 mg/kg	Mouse	+		+	+		M–S	6
	300 mg/kg	Mouse		+		+		S	128
	1 mg/day	Mouse			+	+		S	222
	1.8 mg/wk	Mouse			+	+		S	342
	1–100 mg/kg/day	Mouse			+	+		N–S	109, 110
	80 mg/kg	Mouse			+		+	M	201
	5 mg/day	Rat			+	+		N	161
	2.5–20 mg/kg/day	Rat	+		+	+	+	N–S	97, 362, 365
	25 mg/kg	Rat			+	+		N–E	74
HN2-HN3	1 mg/day	Rat			+	+		N	161
Melphalan[d]	50% of LD50/day	Mouse	+		+	+		M–S	433
Mechlorethamine[e]	0.4 mg/kg/day	Rat	+		+	+		E–M	362
Methylglyoxal-bis-guanylhydrazine	100–150 mg/m²	Man			+	+		N–S	192
1-Methyl-2*p* (isopropylcarbamoyl) benzylhydrazine	124 mg/kg/day	Man			+	+		S	223
	24 mg/kg/day	Mouse	+		+	+, *in vitro*		M–S	12
	370 mg/kg	Mouse	+			+		S	13
Nitrogen mustard N-oxide	5 mg/kg/day	Rabbit	+			+		S	206
Thio-tepa	5 mg/day	Rat			+	+		M	161
Triethylamine	0.5–1.5 mg/kg/day	Mouse	+		+	+		N–S	103

[a] Drug doses may have been given more than once to give the daily dose indicated, daily injections extended for various periods of time.

[b] Abbreviations: severe suppression of immune response, S; mild suppression, M; no effect, N; and enhancement, E.

[c] Endoxan, Cytoxan.

[d] L-Phenylalanine mustard.

[e] NH_2.

TABLE 2

Effect of antibiotics on the immune response

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	Primary	Secondary		
Actinomycin D	5 μg/day	Mouse			+	+		N-M	222
	600 μg/kg	Mouse			+	+		M	157
	7-14 μg	Mouse			+	+	+	S	216
	600 μg/kg	Mouse			+		+	S	157
	35-40 μg	Rat	+	+	+	+		N-S	2
Cetophenicol	150-300 mg/kg/day	Mouse	+		+	+		E-S	142a
Chloramphenicol	50-100 mg/kg/day	Mouse			+	+		N	129
	150-300 mg/kg/day	Mouse	+		+	+		S	142a
	300 μg/ml	Rabbit			+		+, *in vitro*	M	244
Mitomycin C	4-200 μg/ml	Rabbit	+			+, *in vitro*		N-S	112
	0.05-0.5 mg/kg/day	Rat			+	+		M-S	350

See table 1 for symbols.

TABLE 3

Effect of a folic acid antagonist on the immune response

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	Primary	Secondary		
Methotrexate	15 mg/day	Guinea pig			+		+	S	336
	10-100 mg/kg	Mouse	+		+	+		M-S	337
	2-20 mg/kg	Mouse			+	+		M	55
	2 mg/kg/day	Mouse			+	+		M	129
	0.75 mg/kg/day	Rat			+	+		N-M	362, 365
	0.25-0.5 mg/kg/day	Rat			+	+		S	97
	1.25 mg/day	Rat			+	+		S	161
	50-75 mg/5-7 days	Man			+	+		E-S	409
	9-25 mg/m²	Man			+	+		M-S	192
	240 mg/m²	Man	+			+		S	297
	240 mg/m²	Man	+				+	M	297

TABLE 4

Effect of purine and pyrimidine analogues on the immune response

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	Primary	Secondary		
2-Amino-6-benzylthiopurine	10–100 mg/kg/day	Mouse	+		+	+		N-S	103
2-Amino-6-hydroxy-8-phenylpurine	2–150 mg/kg	Mouse			+	+		N	153
2-Amino-6-mercaptopurine	2–75 mg/kg	Mouse			+	+		N	153
Arabinosyl-6-mercaptopurine	60 mg/kg/day	Mouse	+	+	+	+		N	228
	10^{-4}–5×10^{-6} M	Mouse			+	+, *in vitro*		N-M	229
Azathioprine[a]	3 mg/kg/day	Man			+	+		E	409
	10–200 mg/kg/day	Mouse	+		+	+		N-S	103
	33–100 mg/kg/day	Mouse			+	+		N-M	129
	10–80 μg/ml	Rabbit			+		+, *in vitro*	M-S	244
6-Azauridine	180–270 mg/kg/day	Mouse			+	+		N-M	129
	10–1000 μg/ml	Rabbit	+			+, *in vitro*		M	112
5-Bromo-2′-deoxyuridine	33–120 mg/kg/day	Mouse			+	+		N-M	129
	8–1000 μg/ml	Rabbit	+			+, *in vitro*		M-S	112
Cytosine arabinoside	20 mg/kg/day	Dog			+	+		S	171
	2 mg/kg/day	Man	+			+		S	297
	2 mg/kg/day	Man	+				+	M	297
	20–180 mg/kg/day	Mouse			+	+		M-S	129, 170
	2500 mg/kg	Mouse	+		+	+		N-S	182
Deoxycytidine	80–160 mg/kg/day	Mouse			+	+		N	129
α-D-2′-Deoxythioguanosine	30 mg/kg/day	Mouse	+	+	+	+		M	228
5,6-Dichlorobenzimidazole riboside	3.2–320 μg/ml	Rabbit	+			+, *in vitro*		N-S	112
6-(2,2-Dimethylhydrazino) purine	80 mg/kg/day	Mouse	+	+	+	+		N	228

TABLE 4—*Continued*

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	Primary	Secondary		
5-Fluoro-2′-deoxyuridine	10–15 mg/kg/day	Mouse			+	+		M	129
	1–8 mg/day	Mouse	+	+	+	+		E–S	289
	2 mg	Mouse	+			+		E	290
	10–1000 μg/ml	Rabbit	+			+, *in vitro*		M–S	112
	15 mg/kg/day	Rat	+		+	+		E	362
5-Fluorouracil	20–40 mg/kg/day	Mouse	+	+	+	+		N–S	103
5-Iodo-2′-deoxycytidine	300 mg/kg/day	Mouse			+	+		N	129
5-Iodo-2′-deoxyuridine	100–300 mg/kg/day	Mouse			+	+		N–M	129
	80–1200 μg/ml	Rabbit	+			+, *in vitro*		M–S	112
6-Mercaptopurine	300–2000 mg/m²	Man	+			+		N–S	191
	75 mg/kg/day	Mouse	+			+		M	228
	24 kg/mg/day	Mouse	+		+	+		M–S	12
	10–70 mg/kg/day	Mouse	+	+	+	+		N–S	103
	50–75 mg/kg/day	Mouse			+	+		N–S	129
	50–800 μg/ml	Rabbit	+			+, *in vitro*		N–S	112
	6 mg/kg/day	Rabbit			+	+		S	351
	10 mg/kg/day	Rabbit	+			+		E	80
	104–250 μg/ml	Rabbit			+		+, *in vitro*	N–S	244
	20–40 mg/kg/day	Rat			+	+		N	97
6-Methylthioinosine	25–50 mg/kg/day	Mouse			+	+		M–S	228
β-L-Ribosylmercaptopurine	40 mg/kg/day	Mouse	+	+	+	+		N	228
6-Thioguanine	1–6 mg/kg/day	Mouse	+	+	+	+		N–S	103
	40 mg/kg	Mouse			+	+		M	40
	40–1000 μg/ml	Rabbit	+			+, *in vitro*		N–S	112
	5 mg/kg/day	Rat			+	+		S	97

• Imuran.

TABLE 5

Effect of steroids on the immune response

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	1°	2°		
Cortisol	0.004–40 μM	Mouse	+			+		N-S	394
Cortisone acetate	10–150 mg/kg/day	Mouse			+	+		M-S	109, 110
	400–500 mg/kg	Mouse	+		+	+	+	M-S	117
Estradiol	0.025–2.5 mg	Guinea pig	+		+	+		N-S	420
Estrone	0.25–2.5 mg	Guinea pig	+		+	+		M-S	420
Prednisolone	2.5 mg/kg/day	Rabbit			+	+		S	392

TABLE 6

Effect of miscellaneous agents on the immune response

Agent	Dose	Animal	Time of Treatment Relative to Initiation of Immune Response			Type of Response		Reported Effect	References
			Before	Same	After	Primary	Secondary		
Acriflavine	2–5 mg/kg/day	Rabbit	+		+	+		S	355
3-Acetyl-5-(4-fluorobenzylidine)-4-hydroxy-2-oxo-2:5-dihydrothiophen	2.5–10 mg/kg/day	Rat			+	+		M–S	97, 142
Bayer E39 2,5-*bis*(1-aziridinyl)-3,6-*bis*(2-methoxyethoxy)-*i*-benzoquinone	2–7 mg/kg/day	Mouse	+	+	+	+		N–S	103
Bayer 17737 2,5-*bis*-ethyleneimino-3,6-*bis*, acetamino-1,4-benzoquinone	0.5–2 mg/kg/day	Mouse	+	+	+	+		N–S	103
Cinanserin and derivatives	25 mg/kg	Mouse			+			N–S	232
Colchicine	0.04–1.2 μg/ml	Rabbit	+			+, *In vitro*		N-S	112
	1–1.8 mg/kg/day	Rabbit	+	+	+	+		E	213, 417
7,12-Dimethyl-benz-[α]-anthracene	30 μg	Mouse	+			+		N-S	24
9,10-Dimethyl-1,2-benzanthracene	60–1500 μg	Mouse	+			+		N-S	25 446
Epsilon-amino caproic acid	300–2000 mg/kg/day	Rabbit			+	+		N-S	340, 392
Ethidium bromide	1.1–111 μg/ml	Rabbit	+			+, *In vitro*		N-S	112
Endotoxins	66 μg	Rabbit		+			+	M	447
Indomethacin	4 mg/kg/day	Rabbit			+	+		M	392
Phytohemagglutinin	0.5 mg	Mouse	+		+	+		N-S	397
	0.5 mg	Mouse	+				+	M	397
	10 mg/kg/day	Mouse	+			+	+	N-S	329
	1.6 mg	Mouse	+		+	+	+	N-S	158
	4 mg/day	Mouse	+			+		S	217
	4 mg/day	Mouse	+				+	M	217
	4 mg/day	Rat	+			+		M	217
	4 mg/day	Rat	+				+	M	217
Potassium cyanide	10^{-1}–10^{-3}M	Mouse			+	+, *In vitro*		S	39
Sodium 6-acetamidohexanoate	300 mg/kg	Mouse			+	+		S	161a
Thalidomide	12.5–35 mg/day	Mouse	+			+		M	306
	50–100 mg	Rabbit	+		+	+		M	175
	50–100 mg	Rabbit	+		+	+, *In vitro*		N	175
Trenimon	0.05–0.3 mg/kg/day	Mouse	+	+	+	+		N-S	103
Vinblastine	0.1–0.2 mg/kg/day	Rat			+	+		M-S	5
Vincristine	0.025–0.2 mg/kg/day	Rat			+	+		S	5