CHEMICAL SUPPRESSION OF THE IMMUNE RESPONSE

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ABBREVIATIONS AND SYNONYMS

BGG	bovine gamma globulin
BSA	bovine serum albumin
HN2	nitrogen mustard, mechlorethamine, bis(2-chloroethyl)methylamine
HSA	human serum albumin
6-MP	6-mercaptopurine
MTX	methotrexate, amethopterin, 4-amino-10-methylpteroylglutamic acid
TG	thioguanine
B.W. 48-80	polymer of *p*-methoxyphenethylmethylamine and formaldehyde
B.W. 50-197	2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine
B.W. 50-276	2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine
B.W. 57-322	azathioprine, "Imuran," 6-((1-methyl-4-nitro-5-imidazolyl)thio)purine
B.W. 57-323	2-amino-6-((1-methyl-4-nitro-5-imidazolyl)thio)purine
busulfan	"Myleran," 1,4-dimethanesulfonoxybutane
melphalan	"L-sarcolysin," *p*-(bis(2-chloroethylamino))-L-phenylalanine

I. INTRODUCTION

The discovery and characterization of "immunological tolerance" during the past decade and a half has led to an increased depth of penetration not only into the immune process and its core, the recognition of self, but also into many ancillary regions such as organ transplantation and "autoimmune" disease. This is an area in which there is great activity currently, with the consequence that a review such as this is likely to be somewhat out of date however fast it appears. Nevertheless it is worthwhile to attempt a summary of the present status of one of the newer segments of the field because of the breadth of its prospective applications. This review will deal primarily with "drug-induced immune tolerance" and related topics which involve the newly discovered "anti-immune" effects of the antimetabolites. These compounds act primarily during the interval between the introduction of antigen and the appearance of antibody (inductive period), in contrast to previously known agents, such as radiation, radiomimetic drugs, and steroids, which are most effective when given before the antigenic stimulus. Somewhat anomalously the antimetabolites appear to interrupt or suppress various "autoimmune" diseases. The full extent of their activities and their limitations remains to be defined, but it seems appropriate at this time to attempt to put these activities into perspective by comparing them with those of previously investigated agents. To this end, the review will be organized in terms of biological systems rather than by summaries of the effects of individual chemical compounds. An attempt has been made to deal in detail with the literature concerned with antimetabolites; only selected references to other topics will be provided.

"Immunological tolerance" may be described as an atypical state of unresponsiveness to an antigenic stimulus, that is to say that the tolerant individual fails to respond or responds very poorly to an antigenic stimulus which gives rise to good responses in the majority of individuals of the same genus and species. This is a rather broader characterization of the term than was intended by those who used it originally to describe the state of the recipient of a (skin) homograft which was retained. Such breadth, however, is required to deal adequately with the spectrum of phenomena which merge one into the next, without obvious discontinuities, leading further and further away from the original concept.

The beginnings of the current concept of immunological tolerance may be said to date from the observations of Owen (257) on chimerism in twin cattle. Most such twins carry erythrocytes of the other member of the pair throughout life, presumably due to the exchange of hematopoietic tissues which occurs as a result of extensive cross circulation between dizygotic twins in embryonic cattle. Such twins accept skin grafts from each other, but reject grafts from third parties in a normal way (4, 32). The observations of Owen led Burnet and Fenner (47) to propose that the recognition of self is not genetically determined, but is acquired during ontogenesis when immunological competence develops in the continuing presence of autoantigens.

Immunological tolerance was soon produced experimentally. Billingham, Brent, and Medawar injected lymphoid cells of CBA-strain mice into fetal and later into neonatal mice of strain A. The cells were accepted, and such mice accepted any later graft of CBA tissues (28, 30). Similarly Hašek produced parabiosis between chicks in the shell and found that, after hatching and separation, the chicks were tolerant of each other's red cells (146, 147) and skin (148). The induction of tolerance by means of the perinatal injection of cells from the putative donor of a graft is now an established procedure.

Immunological responsiveness can be transferred in the same way as unresponsiveness. If to the graft-host complex of CBA skin on tolerant A mice there is added a complement of lymphatic cells of adult A mice or, better, of A mice previously immunized to CBA skin, the graft will promptly be rejected (29). Such experiments appear to have established that immunological tolerance is due to a failure (or repression) of the recognition mechanism and not to any drastic change in the mechanism for antibody production *per se*. The latter point will be discussed below in connection with tolerance to nonliving, purified antigens.

The presence of living, immunologically competent cells of one line, in tolerant individuals of a second line, may bring an additional complication in the reaction of graft *vs.* host which has variously been termed "homologous," "secondary," "wasting," or "runting" disease (26, 27, 182, 351). In this case, although the host is tolerant to the graft, the cells of the graft respond to the host's tissues as if they were foreign (as indeed they are to these cells) and severe wasting and death of the host ensue. It is only, therefore, with immunologically incompetent (usually embryonic) cells that mutual tolerance and chimerism are the regular result. Tolerance of the host by the grafted cells may occur under some circumstances (see, *e.g.*, 36).

The induction of tolerance through parabiosis can be interpreted as a special case of tolerance in the chimeric state. Parabiosis indeed gives no guarantee that tolerance will be induced, but may result in sensitization to a subsequent or concurrent graft (94, 363). However, parabiotic union for various periods of time may condition the partners to the acceptance of skin grafts, renal grafts, and even entire extremities, which otherwise would have been rejected (180, 217–219, 247, 284, 304, 312). In many instances the tolerance appears to be the result of a competitive replacement of the immune system of the host by those of the parabiotic partner (247) and is accompanied by "parabiotic" or "wasting" disease (247, 284). However, the induction of tolerance to skin grafts from F_1-hybrids in parental strain mice after parabiotic union of hybrid and parent demands a somewhat different interpretation (284). Thus, in the absence of an unanticipated attack of hybrid against parental cells (see, however, 73), it would be necessary to assume that tolerance *per se* had been induced in the parent as a result of the large amount of antigen presented during parabiosis (219, 284). This interpretation would be compatible with the growing evidence, to be presented below, that immunological responsiveness may be repressed by large amounts of an antigen which, in smaller quantity, may give rise to a re-

sponse (36, 139, 225). A further complication arises through apparent qualitative differences in antigenic stimuli, *i.e.*, small differences in the histocompatibility genes of mice are more easily overcome than large differences (139).

Immunological tolerance can be induced to nonliving, as well as living, antigens by perinatal injections (173, 339, 347, 360). However, such tolerance is of finite duration, which is dose-dependent (133, 310). Thus, the alteration in the recognition mechanisms of the host is not necessarily permanent. Moreover, animals that have lost tolerance respond to a later challenge like immunized animals, *i.e.*, by a "secondary" or "anamnestic" reaction (310). Tolerance, however, can be prolonged by "maintenance" injections of antigen (339). This suggests that the maintenance of tolerance is dependent on the continuing presence of the antigen. In animals bearing grafted cells this requirement is automatically provided through the continuing presence of these cells. With nonliving antigens the supply must be renewed from time to time. The greater the degree of immunological competence of the host, the more antigen will be required to produce the unresponsive state. Thus, immunological tolerance merges into "immunological paralysis," a state of unresponsiveness that is inducible with very large doses of a purified antigen, even in adult animals (86, 106).

The chief purpose of this review is to consider means by which adult, or at least immunologically competent, animals may be rendered unresponsive to antigens through the introduction of a third agency, in the form of a drug, into the host-antigen complex. The effects of drugs, in a sense, were forecast by experiments with radiation. Large but sublethal doses of radiation render an animal immunologically incompetent, probably through depletion of the lymphoid tissues, which are the chief sites of the immunological response. Thus when radiation is followed by large doses of protein antigens, the immunological response is feeble or absent, and animals so treated may remain unresponsive for prolonged periods (86). It is clear that X-radiation is more effective when it precedes the administration of antigen by a day or two than when it is given later (18, 335, 337).

Similarly, chimerism, with all the consequences implicit in this state, has been achieved in adult animals by means of doses of radiation, near or above the usual lethal range, followed by homologous or even heterologous grafts of bone marrow or splenic tissue (235, 252, 351). Such animals will accept grafts that are isologous with the donor marrow cells (65, 210, 235). They can be made (with lethal results) to reject the donor marrow by presensitization to it, or "adoptively" by the injection of normal or graft-sensitized lymphoid cells that are isologous with the host (57, 65).

A considerable literature exists on the use of radiomimetic drugs to suppress the immune response. In general, these drugs simulate radiation in acting more strongly when they are given before, rather than after, the introduction of antigen (18, 19, 208). Much of this literature will be dealt with below in the consideration of specific immunological situations.

Cortisone, similarly, may have profound effects on the immunological response (111, 112, 184, 212, 335). Like X-ray, cortisone appears to exert maxi-

mal effects when its administration is begun before the administration of antigen, and may have little activity when given after the antigen (20). Nevertheless, cortisone may have considerable influence on established immunity (113). It has rather profound effects on homograft and heterograft rejection (348) and has been used extensively to suppress the rejection of human neoplasms in heterologous hosts (349, 375).

II. INDUCTION OF TOLERANCE TO HOMOGRAFTS BY DRUGS

A. Conditioning by injection of viable cells

Immunological tolerance to a graft may be induced through the prior treatment of the host with viable cells that are either isologous[1] or compatible with the graft. In some instances, as discussed above in connection with radiation, the treatment clearly leads to a chimeric state, in which the host's immune systems are replaced more or less completely by cells which would be expected to be tolerant of the subsequent graft. Often, however, the extent of replacement of the host's immune apparatus is conjectural. It is perhaps worth while, therefore, to treat such experiments separately from those in which the antigens employed are incapable of self-perpetuation, for experiments of the latter type are less subject to ultimate reinterpretation.

The induction of tolerance in mice to subsequent skin and tumor grafts through pretreatment of the host with amethopterin (methotrexate, MTX) together with spleen and thymus cells of appropriate origin was demonstrated by Uphoff (354, 355). It was necessary, in order to induce the tolerant state, to administer both the drug and the cells at a high level for a considerable period of time. With the tumor system, success was achieved across an apparent histocompatibility barrier at the H-2 locus.[2] Similarly, McLaren (226) reported subsequent skin graft tolerance when donor spleen cells were injected repeatedly into mice during treatment with 6-mercaptopurine (6-MP). An attempt to demonstrate the presence of host lymphatic cells in these animals gave results which were inconclusive. Graft tolerance was achieved only when donor and recipient strains were from the same H-2 group. The results when spleen cells were administered contrasted sharply with the effects of 6-MP alone, which produced only modest increases in homograft survival. Šterzl (320) had attempted similar experiments in rabbits with negative results; however, his

[1] Isologous, isogenic: derived from the same inbred strain. There exist some confusing differences in the use of prefixes, such as "iso," "homo," and "auto," between immunologists and workers concerned with transplantation (*cf.* 238). This review employs the nomenclature of transplantation throughout, *i.e.*, "auto" to mean "self," "homo" as "derived from the same species," and "iso" as "derived from the same inbred strain."

[2] H-2 locus. In the mouse, 14 or more genes at different loci are concerned with homograft reactions, and are termed "histocompatibility" genes. The histocompatibility-2 gene (H-2) is notable because its position within a group on the same chromosome has been localized, and because its effect on the red cell surface has permitted a rapid classification of its allelic modifications by serological means (260a). An H-2 "barrier" is said to exist between strains which possess different H-2 alleles.

dosage of 6-MP (2 mg/kg) probably was too low to constitute an adequate trial.

Attempts to apply this principle to the problem of suppression of renal homograft rejection, however, have met with essentially no success. Mannick *et al.* (215) treated dogs with 6-MP and various sources of cellular antigens, such as lymphoid cells, buffy coat, and splenic homogenate, and subsequently transplanted kidneys from the same donor. The kidneys were rejected similarly to those of untreated controls, essentially as a primary (first-set) rejection. In contrast, in a second series of dogs which had been given lymphoid cells without 6-MP, the subsequent renal graft was rejected by a secondary (second-set) reaction. This result is very similar to that obtained by Woodruff (373), using homologous spleen cells in mice with irradiation, 6-MP, or chlorambucil. In the absence of treatment the mice were sensitized with the result that a later skin graft was rejected by a second-set reaction, but with treatment neither sensitization nor tolerance occurred.

Calne and Murray (53) had reported that blood transfusions from the kidney donor possibly accelerated rejection. Later Calne *et al.* (52) studied the effects of an additional antigenic stimulus in the form of reticuloendothelial cells in conjunction with azathioprine (B.W. 57-322). In a series of 8 animals so treated there was one long-term survivor (>5½ months), a score not markedly different from those of other subseries without the added cellular material.

B. Direct suppression of homograft reaction

An effort is made to introduce each discussion of the effects of drugs on the homograft reactions to specific tissues with background material just sufficient for a reasonable perspective. For a more complete treatment of the subject the reader is referred to the proceedings of the various Homotransplantation Conferences (66–69, 280) and to the admirable review by Hašek *et al.* (150).

1. The transplantation of *skin* from one site to another on an individual (autograft), between identical twins, or from one individual to another in a highly inbred strain (isograft), is followed by healing, revascularization, and acceptance within a short period (*ca.* 8 to 11 days in mice). Genetic differences between donor and host are reflected in a more or less prompt rejection of the graft, which depends mainly on genetically controlled histocompatibility factors (11, 14, 21, 71, 228) and sex differences (34, 95). Rejection is accompanied by immunization, as demonstrated by the more rapid and dramatic second-set reaction when a second graft from the same, or genetically similar, donor is applied, and by the adoptive (35) and passive (149) transfer of such immunity. The antigens from skin appear to be composed essentially of insoluble lipoproteins in which the specificity is believed to reside in the lipid component (80). Skin homograft rejection is accompanied by the rapid proliferation of cells in regional lymph nodes (5, 64, 306) and to a lesser extent in the spleen (5, 64, 263), but appears to involve only minimally the plasmocytes, which ordinarily are associated with the production of soluble antibodies (5). The rejection phenomenon is believed to be primarily cell-borne, *i.e.*, an invasive attack by cells of lym-

phatic origin on the graft (150), but evidence has been advanced that humoral antibodies play a role in this phenomenon (191, 246).

Tolerance to skin homografts can be induced by prior conditioning of the recipient: with bone marrow cells given neonatally (31) or after X-irradiation (210), with splenic cells given similarly (139, 217), or by parabiotic union (217, 218, 247); but it fails to develop with neonatally given donor leukocytes in puppies (118). However, radiation chimeras, produced by a heterologous bone marrow graft (rat to mouse), rejected donor skin, while retaining donor cells of bone marrow origin, a finding that directs attention to the nonidentity of cellular antigens from different tissues (285, 308). This phenomenon has been termed "restricted tolerance" (36).

In the preceding section, a few examples have been discussed of drug-induced tolerance to skin homografts that involved the injection of viable cells of reticuloendothelial origin. A number of attempts have been reported directly to influence the survival of skin grafts through treatment of the host with drugs. Radiation has significantly prolonged the survival of skin grafts in rabbits (84) and mice (59). Cyclophosphamide was active in rabbits (179) and mice (331, 332), but mechlorethamine (HN2) and melphalan were inactive (1, 227, 332). The combination of X-irradiation and urethane was significantly better than either alone in mice (59). Amethopterin was inactive in the rabbit (229) but somewhat active in mice (286) and dogs (153, 341). 5-Fluorouracil was inactive in the rabbit (229), but had some effect in mice (332) and on the transplantation of fish scales (137). 6-Mercaptopurine extends the neonatal tolerance to skin grafts in chicks (286) and, to some extent, to fish scale transplants (137) and skin transplants in rabbits (115, 229–231, 279, 299, 301), rats (340), and mice (226, 332), although negative results also have been reported (85, 172, 229, 279, 286).

Evidence supporting a positive effect of 6-MP on the homograft reaction in rabbits is found in observations on the effects of the drug on the morphologic responses in lymph nodes proximal to the graft (6). The main effect was the inhibition of the proliferation of the "hemocytoblasts" which ordinarily occurs in response to the graft. Rejection did not take place so long as these cells were repressed, but occurred promptly when these cells "escaped" from inhibition. Somewhat similar effects have been reported when cortisone was used locally, but not when it was used systemically (305). In mice, the results are influenced by the relationship of donor and recipient with respect to histocompatibility genes (226). The one reported test in guinea pigs was negative (172). The mercaptopurine derivative, azathioprine, gave slight prolongation in the dog (153). Cortisone and ACTH permitted increased survivals of skin grafts (33, 364), but these observations do not appear to have been explored extensively. Various additional substances had some activity: thioguanine plus azaserine in mice (332) and B.W. 48–80 (a liberator of histamine which appears to act primarily on mast cells) in rabbits (40). Antitumor agents, such as mitomycin and Actidione in mice (332), 8-azaguanine in rat and rabbit (229), and 6-methylthiopurine (229), busulfan, and aminopterin (115) in the rabbit, were inactive.

Radiation alone had some effect (84), which appeared to be greater when it preceded grafting (98). The effects of carcinogens are interesting but not altogether consistent. Thus, Rubin has reported striking tolerance to (DBA/2 x C3H)-F_1-skin by DBA/2 recipients when the latter were treated with methylcholanthrene (285). These effects were prolonged when the hosts were given MTX (285) but nullified by 6-MP (286). However, Lindner (202) found increased survival of skin grafts with methylcholanthrene only when grafts were applied near the time of appearance of tumors (202), and then only in systems, like Rubin's, with small antigenic differences between host and graft. Perhaps this somewhat resembles the prolonged survival of skin grafts in cancer patients (125, 126).

On the whole, reports of positive effects by drugs must be given greater weight than negative reports, since many of the latter are suspect because of inappropriate protocols. In particular, one is struck by the failure of a number of workers to determine the maximum tolerated doses of the drug in question in the species employed. Reports of negative effects by homeopathic doses of drug are meaningless. However, many of the investigations which are reported above have been carried out with due regard to all the known factors, and, although prolongations of survival of skin homografts have been demonstrated, in general these have not been very great and are in striking contrast to the effects on renal homografts to be discussed next. Prolonged tolerance has not been achieved in any instance with skin grafts, and, even where significant prolongation was achieved, this had essentially no influence on the rejection of subsequent grafts by a second-set mechanism (84, 299, 301).

2. Kidney transplants between dogs are regularly rejected within a very short period of time; 11 (83), 14 (93), 15 (376), and 20 days (49) have been reported as the longest survival times in typical series. Rejection is slower in puppies than in adults and perhaps is more subject to modification (198). Thus Gomboš *et al.* (134) obtained three long-term survivors when puppies were subjected to exsanguination-transfusion in the first days of life and, after an interval of months (3, 3, and 8 months, respectively), received renal transplants from the same donor. It may be significant that when this interval was longer (10 to 13 months) the grafts all were rejected. The results of Gomboš may be contrasted to those of Egdahl and Hume (94), who found that cross-circulation between adult dogs for periods of 5 minutes to 3 days induced only immunization to subsequent grafts. Similarly, Wheeler and Gomez (363) reported that short periods of cross-circulation actively immunized dogs to transplants introduced 9 days later. Kamrin (180), on the other hand, was able to get "takes" of renal transplants in rats by means of parabiotic union preceding or simultaneous with the renal graft. Other attempts to induce tolerance to renal transplants through prior injections of viable cells have been discussed in Section IIA.

The rejection of a renal transplant in man follows the same pattern as in the dog (382). It is, however, much more difficult to document the expected survival when donor and recipient are unrelated, because of the paucity and relative obscurity of such data. The success of transplants between identical twins (245,

374, 382) has led not only to the establishment of surgical procedures and management, but also to the investigation of means of modifications of the homograft reaction when genetic differences exist between donor and host. Using X-irradiation and corticosteroids, Merrill *et al.* (233) transplanted a kidney between nonidentical twins successfully. Generally, however, total body irradiation of a magnitude sufficient to suppress the homograft reaction (600 to 700 r) leads to insurmountable problems of hemorrhage and infection in the absence of surviving bone marrow grafts (82). By the selection of closely related and closely matched donors Hamburger (143) has achieved three long survivals in a series of six such cases when preoperative X-ray (430 to 460 r) was given. Since the introduction of antimetabolites, particularly 6-MP and its derivatives, the use of these with or without irradiation has given encouraging results in a number of cases.

Baker *et al.* (8) had reported in 1952 some prolongation in the functioning of renal homotransplants of dogs by means of mechlorethamine, cortisone, and splenectomy. Recently, highly significant, drug-induced prolongations were observed by Calne (49, 50). After the report of 6-MP-induced tolerance to human serum albumin in rabbits by Schwartz (298), Calne treated dogs with 6-MP, beginning at the time of transplantation and continuing as maintenance therapy at approximately maximum tolerated dosage. Considerably increased retentions of grafts were obtained (up to 47 days), but these appeared to require the steering of a very narrow course between the Scylla of toxicity and the Charybdis of rejection. The experience of others with this drug has been similar. Thus, Zukoski reported an average survival of 23.7 days of grafts in dogs treated with 6-MP as against a control average of 7.5 days (378–380) and Pierce reported 15 of 51 homografts to have survived beyond 15 days (265). Among the latter was one very long-term survivor. This graft, a third kidney transplanted into the neck of the animal, survived excision of the animal's own kidneys and subsequent (after 8 months) discontinuance of the 6-MP. Fourteen months after transplantation, the kidney appeared to be normal, a conclusion which was supported by renal biopsy, normal blood urea nitrogen values, and normal clearances of creatinine and *p*-aminohippuric acid. The author concluded that tolerance to a transplant can be induced in the adult animal if the immune response of the host can be suppressed for a sufficient period of time (264). Significant inhibition of renal homograft rejection in rabbits by 6-MP also has been reported (249).

Several other antimetabolites have been studied in connection with renal homografts in dogs. Thus Zukoski, Lee and Hume (381) reported that 8-azaguanine, 6-azauracil, azaserine, MTX, and 5-fluorouracil failed to prolong survival (381), but 8-azaguanine possibly could serve to replace part of the 6-MP dosage (379). The combination of 6-azauracil and urethane (161) gave significant but not striking prolongation (376). Prednisolone (30 mg/day) produced prolonged survival in 1 of 8 animals (376).

Among the drugs directly related to 6-MP, thioguanine and its S-imidazolyl derivative, B.W. 57-323, did not prevent rejection in bone-marrow-depressing doses (51, 53); azathioprine was easier to control as regards bone marrow de-

pression and at least as effective as 6-MP in inhibiting rejection (51). The series with azathioprine included one long-term survivor, the longest in Calne's experience to that time (51). Zukoski also found significant prolongation with azathioprine; one dog in a series of 7 lived for 148 days (376). This author had found 6-methylthiopurine also to have significant activity in most of a series of 8 dogs (376), as did the riboside of 6-MP (381). This series included one long-term survivor which still is retaining the transplanted kidney more than a year after operation (377) and 6 months after discontinuation of treatment with 6-methylthiopurine.

Meanwhile, Calne, Murray, and Alexandre have pursued investigations with azathioprine in various regimens and combinations. In 6 of 12 dogs with transplants from nonrelated donors treated with azathioprine alone, rejection occurred in an average of 25 days; in 11 dogs bearing litter-mate homografts rejections were minimal and one long-term survivor was obtained (53). The use of actinomycin C, while ineffective alone (3), appeared capable of aborting incipient rejection in animals receiving azathioprine and thus prolonged survival (3, 52, 53); 10% of such animals survived more than 3 months, and 3 of 104 were alive after 5½ months (52). Cortisone (52), irradiation (52), HN2 (52), and MTX (3) increased the hazards of the regimen but failed to contribute to its effects. Transfusions of donor blood appeared to hasten rejection (52). A combination of azathioprine and azaserine gave superior results (3); all of 16 animals survived more than 20 days, with 3 alive and well beyond 3 months.

The overall picture, then, in renal homograft studies in dogs is one of slow but rather steady progress toward longer suppression of the homograft reaction, with the encouraging finding that when long-term suppression is achieved in the occasional animal, eventual withdrawal of the drug is permissible with apparent tolerance of the graft.

Studies of renal transplantation in man are following closely the pattern established by work in the dog; however, much of this work is too recent to have appeared in the literature. Kuss *et al.* (192) have reported a series of cases in which a variety of regimens has been employed. Irradiation alone, used in 3 cases, resulted in 1 rejection (unrelated donor and recipient), one death from irradiation, and 1 success in a sister-to-brother transplant (the patient died after 5 months, of unrelated causes). In two patients treated with irradiation, 6-MP, and cortisone, rejection was inhibited although donor and recipient were unrelated in each case (192); one slowly rejected the kidney and died at 17 months; the other was alive and well at 12 months (193). The patient who survived for 17 months was irradiated initially with whole body (400 r) and local X-ray (200 r to the spleen). At 7 weeks, beginning rejection was apparent, and at this time additional irradiation (100 r), cortisone, and 6-MP were given. A striking reversal of incipient rejection followed (194). A similar reversal of incipient rejection by treatment with 6-MP was observed by Murray (244). Kuss *et al.* favor a regimen of sub'ethal irradiation followed by transplantation and therapy with antimetabolites.

Several successful transplants of varying duration involving therapy with

azathioprine are known only by personal communications. Three (parent to child) have been treated with azathioprine alone (372); two (unrelated donor and recipient) with azathioprine plus actinomycin C and azaserine (243). In man, as in the dog, it is possible to conclude that considerable progress in renal transplantation is being made with suppression of the immune response by drugs and that the limits have not yet been reached.

3. Bone marrow transplantation usually is practiced not for its own sake, but either to replace the deliberately destroyed marrow of the host or to induce tolerance in the host to the subsequent transplantation of some other tissue or organ of the donor. Some examples of the latter have been discussed in a previous section. Bone marrow transplantation in mice (58, 351) and rats (252) may prevent death from irradiation; however, depending on the genetic differences between host and donor, this may be followed by homologous disease (351, 357). The ultimate aim of many such studies is the cure of leukemia. The problems involved are well illustrated in a paper by Mathé *et al.* (221). A leukemia of the DBA/2 strain of mice (L1210) was inoculated into hybrids (C57BL6 x DBA/2)F_1. Following otherwise lethal X-irradiation, isogenic bone marrow prolonged life but failed to eradicate the leukemia. When a parental marrow (C57BL6) was used, some animals were cured of leukemia, but succumbed to secondary disease. However, the secondary disease could be suppressed, with either MTX or cyclophosphamide, and the whole regimen yielded a significant number of survivors free of leukemia.

"Myleran" (362) and dimethylmyleran (116) produced primarily marrow aplasia, and rodents treated with lethal doses of these agents could be saved by transplants of isologous bone marrow. However, homologous marrow did not take in mice treated with dimethylmyleran (116) and this intolerance of the host toward the graft could not be overcome by treatment with 6-MP or cortisone (116). Nevertheless, success has been reported with similar experiments in dogs. Whereas X-irradiation alone at 900 r was insufficient to permit takes of homologous cells, treatment with either 6-MP or urethane prior to the irradiation was followed by successful marrow transplantation (57). Marrow function that had been destroyed by radiation in dogs was restored by autologous (isogenic) marrow cells (344). Following high levels of irradiation (1200 to 1600 r), infusions of homologous marrow usually permitted survival, but resulted in secondary disease (342); however, the latter could be treated with MTX with considerable success (205, 343).

Man appears to present greater obstacles to the successful transplantation of marrow than do other species, and several authors have reported lack of success following irradiation, either alone (82) or combined with chemotherapy (240, 275). However, Mathé *et al.* (223) feel that late transplants (a month after exposure) result in takes.

4. Lung homotransplants in the dog have exhibited prolonged survival after total body irradiation, bone marrow transplantation, and suppression of secondary disease with MTX (38, 39). In these dogs evidence was obtained of pulmonary function (39), but function possibly was less evident in corresponding homo-

grafts when the host was treated with MTX alone, although rejection was inhibited (39). The survival of a graft appeared to be enhanced when the recipient was pretreated with donor blood together with either X-irradiation or MTX (39). The rejection of lung homografts in dogs also is inhibited by azathioprine (12, 39). Hardy *et al.* (144) reported an average survival of dogs with a single homotransplanted lung of 7.4 days; however, treatment with azathioprine extended the average to 30.4 days (20 dogs), a result which was not improved by the addition of either hydrocortisone or actinomycin C to the regimen. A small and somewhat unsatisfactory series (6 dogs) with MTX gave an average survival period of 13.8 days.

5. Heart transplantation has been investigated fairly extensively in the dog. A few such studies have dealt with direct replacement, but the bulk of the work has used the technique, first described by Mann and associates (214a), in which the aorta and pulmonary artery of the transplant are anastomosed to the carotid artery and jugular vein of the host. In various series, the maximum period of survival of a homograft ranged from 6 to 10 days (275). Reemtsma *et al.* (275) recently reported that this period could be extended significantly by treatment of the host with MTX. One graft survived for 26 days and 10 of 21 transplants survived more than 10 days. Rejection and drug toxicity were implicated about equally among the causes for failure. If it becomes possible, ultimately, to control rejection by means of tolerable doses of a drug, one would predict that new problems will arise, since the heart, probably even more than the lung (see above), may require innervation for its function.

6. Tumor. The role of immunity in the natural control of neoplastic growth is a field of immense complexity and little general agreement (237, 314). The question has been raised legitimately whether one may not, in treating the tumor-bearing subject with agents which have not only antitumor but also anti-immune effects, be working at cross purposes (297). Where the tumor and host are genetically dissimilar it has been possible to demonstrate enhanced tumor growth as a result of suppression of the homograft response. Thus Jackson, Preston, and Henegar have examined the effects of drugs on the growth of the Bagg lymphosarcoma in Wistar and Lewis rats. Under control conditions the tumor was rejected, with survival of the host, in 50% or more of the trials. In the Wistar rats, pretreatment with a variety of agents resulted in enhanced growth of the transplants (158, 268, 269). The effective agents included N-mustard derivatives (such as melphalan and cyclophosphamide), antibiotics (such as miracil D and Actidione), and a combination of thioguanine (TG) and azaserine, although other antimetabolites (such as 5-fluorouracil and 6-MP) were inactive. The effects in Lewis rats were less pronounced; only cyclophosphamide showed significant activity.

Goldin and collaborators have studied a variety of systems which involved strains of leukemia L1210. In what was perhaps the simplest, the leukemia was transplanted into incompatible strains with the result that it regressed and immunized the host. When an amethopterin-resistant line of the leukemia was used, treatment of the host with MTX repressed the homograft rejection, and

the leukemia grew progressively, with ultimate death of the host. Nevertheless, MTX neither interfered with established immunity nor prevented the development of immunity to the sensitive strain of the leukemia (132, 174). Further studies have revealed a "dilution effect" whereby small inocula gave higher lethality than larger inocula (175). This was interpreted to mean that the larger inoculum produced an immune reaction which was not stimulated when the smaller number of cells was used. Treatment with MTX made even the large inocula lethal when a resistant line was used; this effect could be reversed by citrovorum factor (175).

Additional examples may be cited of the suppression of the homograft response to the detriment of the host. Thus, Rubin (283) found that prior treatment with carcinogenic hydrocarbons allowed the growth of tumor grafts in mice which otherwise would have been resistant; however, postimplantation treatment had the opposite effect. Treatment with salicylates permitted the growth of a human melanoma in the hamster and was more effective than treatment with cortisone (307). On the other hand, cortisone encouraged metastasis of sarcoma I of C57 mice, while aminopterin, which had an equivalent effect on the inflammatory response, failed to do so (241). However, treatment of the host with MTX permitted the growth in foreign hosts of the amethopterin-resistant lymphocytic mouse neoplasm P288 (266). Alkylating agents failed to reduce the resistance of rats to a lymphosarcoma, but a combination of X-ray and cortisone resulted in growth of the tumor (293). A black, Walker sarcoma-resistant rat could be made to support the growth of the tumor by treatment of the host with 6-MP (to which the tumor is relatively insensitive) (162). Additional examples of a similar nature have been cited by Koelsche (187).

The relevance of these studies to the problems of human cancer are not at once apparent; presumably, the results would become significant only with the demonstration that immunity to autochthonous tumors has a significant restraining effect. Moreover, there is some evidence for the development of immunity to tumor grafts during effective chemotherapy with immunity-suppressing agents. Thus Goldin, cited above (174), found that immunity to L1210 leukemia developed during therapy with MTX, and Tarnofsky and Stock have put forward evidence that the late regressions of sarcoma 180 after therapy with 6-MP are due to the development of immunity (338). Chemotherapy of hemoblastoses with 6-MP, corticosteroids, busulfan or chlorambucil did not influence a number of indices of immunological activity (236).

7. Secondary disease. Radiation and a variety of chemotherapeutic regimens can induce tolerance in the host to viable cells of reticuloendothelial origin (Section IIA). The resulting chimeras frequently come to an early demise as a result of the graft *vs.* host reaction. Several examples of the therapy of this secondary disease have been described above, coincidentally with other considerations, but it is perhaps pertinent at this point to review a number of studies directed primarily to its repression by means of drugs.

Success in the suppression of secondary disease was first reported by Uphoff (353), who found that mice that ordinarily would succumb to the homograft

reaction after irradiation and bone marrow grafting, could be protected by treatment with MTX. This finding formed the basis of her later work, cited above, in which the folic acid antagonist both induced tolerance and prevented secondary disease. A similar protocol has been followed by Thomas *et al.* (341, 342) using radiation, bone marrow grafting, and subsequent treatment of the secondary disease syndrome with MTX (341, 342) to condition dogs to the reception of skin and lung grafts (38, 39). Mathé also has found that secondary disease in mice can be controlled with either MTX or cyclophosphamide, even in subtherapeutic dosage (220), and this has been advantageous in the treatment of leukemic disease by homologous bone marrow grafting (220, 221).

Chemotherapy does not always work to the advantage of the host. Thus, Schwartz (295) found that prednisone or 6-MP, in therapeutic doses, accelerated the course of the graft *vs.* host reaction. However, smaller doses of 6-MP (10 mg/kg per day), given to hybrid mice either before or during the administration of parental spleen cells, retarded the development of runting. The graft *vs.* host reaction which followed parabiotic union in dogs was enhanced by treatment with azathioprine or MTX (152). Prior irradiation (200 r) of the cross-circulation partner prevented this reaction. A similar interference with secondary disease in mice through prior irradiation of the donor cells was reported by Cudkowicz (74).

Antimetabolites thus may favor either the graft or the host in the graft-host conflict. In view of the importance of timing and dosage (295) to the result, it is premature to attempt to compare the effects of different antimetabolites.

Oliner *et al.* have drawn attention to similarities between runting disease and autoimmune diseases in man (253). Sinkovics (309) has reported that certain potentially malignant lymphoid cell clones can produce a wasting disease in new born mice which includes among its signs a Coombs-positive hemolytic anemia. The relationships among graft *vs.* host reactions, autoimmune disease, and lymphomatous disease remain obscure but appear to offer a promising area for further investigations (77, 182).

III. SUPPRESSION OF RESPONSE TO NONLIVING ANTIGENS BY DRUGS

A. Introduction

Several aspects of tolerance to nonliving antigens have been considered in brief in previous sections of this review. Thus tolerance may be induced by the injection of the antigen 1) perinatally, 2) following X-irradiation of the recipient, or 3) into animals which were thymectomized neonatally. Moreover, tolerance or immune paralysis may be induced in adult animals when the amount of antigen given is very large compared with the minimum immunizing dose. This work has led to the concept that some threshold concentration of the antigen must be attained to repress the immune response—a threshold which rises sharply with increasing immunological competence of the recipient. It seems clear that the antigen must continue to be present to maintian tolerance, although the amounts required may be very small once the unreactive state has

been established. The route and method of administration, the presence or absence of adjuvants, the nature of the antigen and the species being tested all may have profound effects on the results of antigen administration. The sorting out and quantitative evaluation of many of these factors remain for future work. For details, the reader is referred to the reviews already mentioned and to that of Smith (313).

B. Tolerance

A new and fascinating aspect of this picture was revealed by the finding of Schwartz *et al.* (303) that the antibody response of rabbits to bovine serum albumin (BSA) could be completely suppressed when 6-MP was given during, and for a short time after, the course of immunization. Neither pretreatment, nor treatment after the response had developed, had a significant effect on antibody production. In similar experiments with human serum albumin (HSA), treatment with 6-MP at a higher dosage resulted in blockade of the primary response but only slightly modified the secondary response of previously immunized rabbits (296, 302). An extension of these investigations showed, in fact, that tolerance to HSA was produced by vigorous treatment with 6-MP during the first presentation of the antigen. A challenging dose of the antigen given a month later failed to elicit a response and, after the lapse of an additional month, a third injection of HSA likewise failed to induce a response. The criterion of specificity of the tolerant state was satisfied by the demonstration that the tolerant animals formed antibody to bovine γ-globulin (BGG) in the normal way when this antigen was given with the second dose of HSA (298).

Goh *et al.* (131), using radioiodinated HSA and human γ-globulin as antigens in the rabbit, have confirmed the finding that treatment with 6-MP blocks the immune phase of elimination of the circulating antigen, but only a small proportion of the animals failed to show a response on rechallenge. The reason for the discrepancy between the results of Goh and those of Schwartz is not at once apparent, since other authors (105, 127, 313) apparently have had no difficulty in producing tolerance to serum albumins in rabbits by this technique, and Smith (313) has reported that tolerance produced in this way has many resemblances to neonatally induced tolerance. The general validity of the phenomenon finds further support, paradoxically, in experiments which contradict one of Schwartz's earlier conclusions. Thus Condie *et al.* (60, 62) have shown that 6-MP can block antibody production in the immunized animal and La-Plante *et al.* (196) found that the secondary immune response to BSA can be suppressed completely in this way. Such animals then fail to react to a third challenge of antigen. To achieve the unresponsive state in previously immunized animals, vigorous treatment with both antigen and drug and the proper timing were required (196). The difference between the effects of 6-MP on the primary and secondary responses thus appears to be quantitative rather than qualitative.

It may be that Schwartz chose an exceptionally favorable model for his work. It is known, for example, that tolerance to BSA in rabbits can be induced by the neonatal injection of doses which are several orders of magnitude lower than

those required in neonatal mice or 14-day chick embryos (313). This suggests that for the induction of tolerance in the rabbit, the threshold dose of antigen of this type may be particularly low. As one deviates more from Schwartz's model, the results of therapy with 6-MP become less decisive. Thus, for example, Robinson and Christian (279), using ovalbumin in Freund's adjuvant[3] subcutaneously in rabbits, found that therapy with 6-MP suppressed the formation of antibodies during the period of drug administration in each of 4 animals tested. However, of the 2 which survived beyond 21 days, one developed a low titer of antibodies spontaneously, while the other responded to a second dose of the antigen. Similarly, Hoyer *et al.* (169) found that the inhibition of antibody to BSA was incomplete when the antigen was given in Freund's adjuvant. Humphrey and Turk (173) reported failure to achieve tolerance to BSA or human γ-globulin in guinea pigs by means of therapy with 6-MP, and Genghof and Battisto reported a similar failure with BGG (127). In part, these results may be attributed to a failure to recognize the large difference in the maximum tolerated dose of the drug in the two species, but a clear species difference in responsiveness also exists.

These reports illustrate a few of the many factors which must be taken into account in relation to the production of the immunologically unresponsive state. It is apparent that the use of 6-MP to induce tolerance in adult animals does not have the universality of application which prevails with perinatal injections of antigen. Nevertheless, as will be discussed below, the suppression of immune responses to a wide range of antigenic stimuli in a variety of species can be obtained with the drug.

C. Delayed hypersensitivity

It seems appropriate to consider briefly the phenomenon of delayed hypersensitivity. There is general agreement that this is an acquired specific reactivity. In the opinion of some workers, delayed hypersensitivity represents a process qualitatively different from that which gives rise to circulating antibodies and is entirely cell-borne. Others hold that the formation of cellular and that of circulating antibodies represent essentially two aspects of the same fundamental process. The latter view is ably supported in the recent excellent review by Karush and Eisen (183), who have put forward the viewpoint that delayed hypersensitivity results when antibodies with a high affinity for the antigen are slowly released and then fixed at the site of deposition of the antigen. The fundamental similarity of hypersensitivity to the antibody response is strongly supported by the work of Turk and Humphrey (352), who showed that guinea pigs made unresponsive to BSA or human γ-globulin, by the perinatal injection of these antigens, were subsequently incapable of developing hypersensitivity reactions to them.

[3] *Freund's adjuvant* consists basically of an emulsion of paraffin oil, lipids, and killed mycobacteria. Many minor variations are used by individual workers. Adjuvants of this type have been found, empirically, to increase the antigenicity of a variety of substances.

D. Individual drugs

Before proceeding to a review of the effects of antimetabolites on the responses of different species to a variety of antigenic stimuli, it may be useful to consider briefly earlier attempts to influence the immune response with chemical agents. Hektoen, as early as 1916, had reported mild depressant effects on antibody production in rabbits and other species through the use of benzene or toluene (154, 155). Similar effects on the development of hemolysins and agglutinins to typhoid bacilli resulted from treatment with arsphenamine or mercuric chloride (350). Hektoen also had reported that mustard gas (*bis*-chloroethylsulfide) significantly delayed the appearance and lowered the liters of lysins and precipitins which resulted from the administration of sheep red blood cells to rabbits (156). He also pointed out that in order to obtain effects it was necessary to administer the substance before or, at the latest, at the time of administration of the antigen, and that the depression of antibody response was never permanent.

When the N-mustards became available, studies on a variety of these agents showed that they possess similar effects. Thus, in short succession, Philips *et al.* (262), Spurr (316), and the Taliaferros (336) had demonstrated interference with a number of immune responses by mechlorethamine (HN2), and Spurr in particular had emphasized the effectiveness of pretreatment. Schwab *et al.* (294) found that X-radiation and HN2 suppressed the formation of circulating antibodies and hypersensitivity reactions to BSA and BGG in rabbits, and Bukantz *et al.* (44) found that HN2 prevented sensitivity reactions to horse serum in rabbits. Benacerraf *et al.* (16) showed that radiation of mice did not interfere with the uptake or breakdown of antigen; hence, its effects could be attributed primarily to the suppression of the antibody response. In man, however, Stoloff *et al.* (327) found that X-irradiation had little effect on the response to diphtheria toxoid. These early observations have been followed by a considerable number of studies in which variants of the nitrogen mustards (such as novoembichin, triethylene melamine, busulfan, and cyclophosphamide) have been used in a variety of species with a considerable number of antigenic stimuli (297). Some of these studies will be mentioned below, particularly in connection with screening tests for substances which suppress the immune response.

At this point the observations of Maguire and Maibach on cyclophosphamide deserve mention, since they represent the closest approach yet reported to immune tolerance through the agency of an alkylating agent (208). These authors treated guinea pigs with cyclophosphamide and, after the second dose, injected a sensitizing dose of egg albumin. When challenged 23 days later, only 4 of 15 treated animals (as compared with 10 of 11 controls) succumbed to anaphylactic shock. The treated survivors subsequently were sensitized and shocked with a different antigen. The authors concluded that the drug had induced a temporary incompetency of the immune apparatus, but it is conceivable that a specific unresponsiveness of considerable duration might have been involved. The same authors reported cyclophosphamide, but not 6-MP, actinomycin C, or

vincaleukoblastine (vinblastine), to suppress delayed hypersensitivity reactions of guinea pigs to dinitrochlorobenzene (209). Stender (318) contrasted the effects of cyclophosphamide with those of X-irradiation on the formation of antibodies to *Brucella* antigens in rats. The former was effective at much the smaller fraction of its LD50; moreover, its activities were relatively independent of the time of administration and were apparent even when the drug was given after antibody production was in full progress.

Salvin and Smith (289) and Uhr and Scharff (356), both using diphtheria toxoid in guinea pigs, found that a delayed hypersensitivity reaction might persist in the face of X-irradiation sufficient to eliminate the development of circulating antibodies.

Salicylates were reported to depress the immune response (333). This was interpreted at one time as an inactivation of antibody (56), but a subsequent study on anti-Rh agglutinins led to the conclusion that, since salicylates were absent from the plasmas tested, the effects were primarily on the formation of the agglutinins (165). This view was supported by the finding of a significant depression by salicylate of typhoid antibodies in man (177), despite an earlier report of the inactivity of aspirin (261)

Sporadic investigations of the activities of the common antibacterial agents on immune responses have given little in the way of consistent effects. Thus, penicillin has been reported not to influence the levels of antibodies in rabbits to sheep erythrocytes (195) but to depress those to antigens of the typhoid group (216) and BGG (324). Dihydrostreptomycin and the tetracyclines also decreased the response to γ-globulin (324). On the other hand, neither penicillin, salicylates, nor sulfadiazine interfered with the development of antistreptolysin and antifibrinolysin titers in cases of hemolytic streptococcal sore throat (274), nor did penicillin influence the antibodies formed in response to pneumococcal infection in rats (311). Chloramphenicol has not been studied as widely as might have been expected on the basis of its interference with protein synthesis in microbial systems, but has been reported by one author to modify the antigenic character of *Salmonella typhosa* in experimental infections in rabbits (103, 104) and to reduce the response to BGG in rabbits (102). Sulfonamides, likewise, appear not to interfere with the development of agglutinins to dysentery bacilli in rabbits (110). Harrison (145) felt that penicillin when given early in infection prevents antibody formation because the rapidity of its action prevents the synthesis of adequate amounts of antigen; later it has no effect on antibody production. The sulfonamide, acting more slowly, does not prevent antigen synthesis and, when it is given late, the titers of antibody may be low because antibody is removed through combination with antigen.

The effects of corticosteroids on the immune response already have been mentioned briefly and no attempt will be made to review the extensive literature on this subject. It is apparent that the effects of corticosteroids may differ widely from species to species (197, 206). In man the formation of circulating antibodies to vaccines and toxoids may be relatively uninfluenced (151, 159, 197), and possibly the same is true in the guinea pig (128) and rat (97), whereas rather profound effects may occur in rabbits (*e.g.*, 37, 112, 129, 212).

Colchicine, when given to immunized animals, appears to diminish precipitin titers, probably through destruction of lymphocytic cells and tissues (101, 117).

It became apparent early in the history of folic acid that a deficiency of this vitamin could lead to severe impairment of antibody production. Thus Little *et al.* (203) found that the agglutinin responses of chicks to multiple injections of *Brucella abortus*, *Pasteurella multocida*, or *Salmonella typhosa* were inhibited, and that the administration of synthetic folic acid reversed this effect. Similar suppressions of responses to *Salmonella* antigens (330, 361) or to human erythrocytes (207) were seen in folic acid-deficient rats. Mice made deficient in folic acid, either by dietary means or by administration of MTX, did not succumb to infection with lymphocytic choriomeningitis (41, 140–142, 200), although the multiplication of the virus did not appear to be inhibited (140, 142). Moreover, mice immune to this virus, when treated with MTX, permitted its passage in conjunction with the neoplasm P288 (266). The evidence suggests that the morbidity and mortality in infections with this virus are associated with the immune response of the host (140), since viremia without or with reduced morbidity can occur not only in amethopterin-treated, but also in prenatally infected mice (140). A different mechanism apparently prevails with regard to the effects of this drug on infections of guinea pigs with vaccinia virus. Here it had been shown that although irradiated animals produced no detectable antibodies, they recovered as fast as did controls (120). Further study showed, however, that such animals eventually developed hypersensitivity reactions (119). Treatment with MTX suppressed not only antibody development but also the delayed hypersensitivity reaction (119), again without influencing the elimination of the virus. Thomas *et al.* (341) have shown that the production of antibodies to attenuated distemper virus in the dog is greatly delayed and reduced when the dogs are treated with amethopterin. However, elimination of the virus apparently did not occur for, when treatment was stopped after three weeks, antibody production became apparent within a few days, and when treatment was continued, antibody production at a reduced rate eventually took place.

The failure of MTX to influence the development of antibodies in rabbits to BGG and sheep erythrocytes was noted by Brooke (43). In guinea pigs, folic acid antagonists prevented the development of delayed hypersensitivity reactions to diphtheria toxoid, ovalbumin (121), or tuberculin (270) and increased the virulence of infection with *Mycobacterium tuberculosis* (270). The above citations serve to illustrate that the full range from ineffectiveness to tolerance or near-tolerance is possible with this agent, depending on the specific circumstances of the test. A fuller appreciation of its potentialities can be gained by a consideration of various studies in which side-by-side comparisons of a variety of agents have been made.

The effects of 6-MP on homograft rejections and in the production of tolerance to serum albumins in rabbits have already been discussed. Following the initial observations of Schwartz (303) its effects have been studied in a variety of immunological systems.

Genghof and Battisto (127) and Salvin and Smith (290) both reported failure of 6-MP to influence the development of hypersensitivity reactions in guinea

pigs. On the other hand, Hoyer *et al.* (171) demonstrated that hypersensitivity in guinea pigs could be suppressed during treatment with 6-MP but developed after the treatment was stopped. Dosage and route of administration of the drug were both important.

Wirostko and Halbert (364a) found that the allergic uveitis which results from the intraocular injection of BSA could be suppressed by treatment of the rabbits with 6-MP.

Šterzl (323) at first found no effect on the production of antibodies to paratyphoid B in adult rabbits but later found this antigen unsuitable because most adult rabbits are immune to it, presumably as a result of natural infections (319). He was able to demonstrate a striking suppression by 6-MP of antibody production in a cell-transfer system. Cells from adult donor rabbits were mixed with heat-inactivated *Bacillus suis* and transferred to young, immunologically incompetent rabbits. When drug was given to the recipients within 24 hours of transfer, the antibody production was markedly suppressed; however, a delay of 72 hours resulted in a significant loss of effectiveness. If the cells were taken from an immunized donor, 6-MP had no effect. Therefore, Šterzl concluded that 6-MP acts only on the inductive phase of the primary immune response (319, 320). Mannick *et al.* found that the production of precipitins to hemocyanin in dogs was completely suppressed by treatment with 6-MP at 5 mg/kg per day for 8 or 14 days. When the survivors were rechallenged at 30 days, however, all gave some sort of response, but this was of the primary rather than the secondary type (215).

Janssen *et al.* reported that 6-MP lowers the resistance of monkeys to both variola and vaccinia viruses, and of rabbits to vaccinia, but not to variola virus (54a, 178, 178a). Antibody levels were significantly depressed in all animals, but in view of the unimpeded recoveries of rabbits unable to form antibodies to vaccinia in the experiments of Friedman (119), it is possible that other factors beyond the suppression of antibody were involved in Janssen's experiments.

Frisch (123, 124) selected 6-thioguanine (TG) as the most potent inhibitor, among the compounds which he tested, of the hemagglutinin response of mice to an inoculum of sheep erythrocytes. By the use of minimal single intraperitoneal doses (2.5 mg/kg) of the drug, he could identify the optimal time of drug administration as 18 hours after the antigen was given; with larger doses (up to 20 mg/kg) effects could be shown when the drug was given between 24 and 72 hours (but not at 2 or 12 hours). Similar effects were obtained with human OD^+ cells. When these cells were used as the primary antigenic stimulus and sheep cells were given at the same time as the drug, a complete block of the formation of the antihuman agglutinins, without any effect on the anti-sheep hemagglutinins, was demonstrable 36 hours later. Frisch argued that this timing suggests that TG produced a metabolic block in the last two-thirds of the induction process, and suggested a number of possible ways in which this might occur (123).

Wolff and Goodman (371) reported that treatment of both patients and rabbits with TG results in a hypogammaglobulinemia and suggested that cells producing γ-globulin are inhibited selectively.

Malmgren (214) reported that the administration of 8-azaguanine reduced the hemolysin and precipitin antibody titers in mice and did not interfere with preformed antibodies, and Dutton *et al.* (88) confirmed the activity of this substance in a system involving isolated spleen cells, but later reported that it lacked specificity in its action (89). The comparative studies to be discussed below suggest that this purine analog is less active than the thiopurines.

Treatment with the methionine homolog, ethionine, reduced complement but not agglutinative or complement-fixing activity in guinea pigs responding to killed *Brucella abortus* cultures (278). β-Thienylalanine, when fed to rats receiving a minimal amino acid allowance, depressed antibody synthesis and caused weight loss (365). The effect on antibody production, but not the weight loss, was corrected by the addition of phenylalanine to the diet. Stavitsky (317) showed that a number of amino acid analogs may interfere with the synthesis of antibody by lymph node cells (from immunized rabbits) *in vitro*, and that these effects are reversible by the appropriate amino acid. These studies also served to document the fact that antibodies are synthesized *de novo* and not derived to a significant extent from pre-existing cellular proteins.

5-Bromodeoxyuridine was a highly effective inhibitor of the synthesis of antibody *in vitro*, using spleen (90) or lymph node (250) cells from rabbits after anamnestic stimulation with ovalbumin (90) or diphtheria toxoid (250). This substance was active over a wide range of concentrations in suppression of the hemagglutinin response of mice to sheep erythrocytes (24).

E. Screening tests

A number of studies, the bulk of which were designed as screening tests for anti-immune drugs, have dealt with the direct comparison of the effects of a variety of drugs on a chosen immune response. Berenbaum (17) administered TAB (typhoid-paratyphoid A and B) vaccine to mice and determined the antibody titers 10 days later. He tested the effects of a variety of materials given at the same time as the antigen. Chlorambucil, pyrimethamine, triethylene melamine, 6-mercaptopurine, and ribonuclease were selected as active. In further work, dose-response and timing relationships were explored in detail. With irradiation and alkylating agents, the greatest effects were obtained when treatment was begun 1 or 2 days before the antigen was administered; irradiation, in particular, was ineffective when given after the injection of antigen. The antimetabolites, 6-MP, pyrimethamine, and MTX, were most effective when given 1 to 2 days after the antigen (18, 19). Among the inactive compounds were 8-azaguanine, 2,6-diaminopurine, hydrocortisone, and urethane.

Šterzl used the transfer system described above to test the effects of a number of drugs. The 3- to 5-day old rabbits, bearing grafts of sensitized lymphoid cells, produced antibodies which reached a maximum titer 3 to 5 days after grafting, although untreated rabbits of this age are immunologically incompetent. Drugs were given daily for 5 days after the transfer of cells (321, 322), and hence were acting primarily during the inductive phase of the immune response. The purine antagonists, 6-MP and TG, caused marked effects, while 8-azaguanine gave an

equivocal effect. Aminopterin produced strong suppression of the response. Among the pyrimidine analogs, only 6-azauridine gave strong effects, while 5-fluorouracil was inactive even at toxic levels; 5-bromouracil, 2-thiouracil, and 6-azauracil were inactive, but 6-azathymine gave an equivocal result. Busulfan caused a marked effect at a toxic level, but was inactive at a lower dose, and chlorambucil was inactive. Colchicine and actinomycin C likewise were inactive.

Procedures using the hemagglutinin response of mice to sheep erythrocytes have been employed to test for the effects of drugs on the immune response. Malmgren (213) had explored such a system in 1952, giving carcinogens in 12 doses beginning a week before the administration of antigen, and other drugs for 5 days from the time the antigen was given. Amethopterin, 2,6-diaminopurine, colchicine, α-peltatin, urethane, triethylenemelamine, and a variety of carcinogens, including methylcholanthrene, dibenzanthracene, and butter yellow, were active.

The relationships among dose of antigen, dose of drug, and the timing of drug administration were explored in detail by Nathan *et al.* (248). In agreement with Makinodan (211), they found that after a single dose of erythrocytes, given intravenously, antibodies appeared 4 days later and the titers rapidly increased to a maximum. In order to show suppression of the response, large doses of antigen were required. The drugs were most effective when treatment was initiated at the time the antigen was given; a delay of even one day appeared to diminish the effect, but maximal effects on the titers at day 12 were obtained when treatment was limited to the 4 days of the induction period. Under these conditions more or less linear dose-response relationships could be shown for a number of drugs (248). The results were generally in good agreement with those of Šterzl (322). Thus, among the purine analogs, marked effects were exerted by 6-MP and TG and their respective S-(1-methyl-4-nitro-5-imidazolyl) derivatives (B.W. 57-322 and B.W. 57-323), while 8-azaguanine was on the borderline of activity (248). 6-Methylthiopurine was active, but 6-propylthiopurine was inactive. Azathioprine was notable for the extent of its dose-positive response relationship, showing activity over a 4- to 6-fold range within the limits of tolerated doses. Among the pyrimidine analogs, 5-aminouracil, 4-thiouracil, and 6-azauracil showed significant but not strong effects, while 5-bromouracil and 5-fluorouracil were inactive. The folic acid antagonists, MTX and B.W. 50-276, caused positive but not very strong responses. Among the alkylating agents, only chlorambucil showed significant effects at tolerated doses. Actinomycin D, but not actinomycin C, was scored as active; however, the activities of both were borderline. In a continuation of this work (24, 161), 5-bromodeoxyuridine had activity at unusually small fractions of the maximum tolerated dose. It and other active pyrimidine nucleosides, such as 4-thiodeoxyuridine, exhibited synergistic effects when combined with the thiopurines. This apparently could be correlated with the cellular responses of different elements of the splenic pulp to the two types of drug (25).

Frisch (122-124) has used a similar sheep erythrocyte-mouse system; the hemagglutinin titers were determined in individual mice with blood obtained by

the orbital puncture technique. Dose-time relationships, worked out primarily with single doses of TG (123), showed the time of maximum sensitivity to be 18 hours after the administration of antigen, while pretreatment was ineffective. In general, the system employed by Frisch appears to be somewhat less sensitive than that of Nathan, but the relative effectiveness of different compounds is similar. Thus, TG was the most active drug, followed by 6-MP, B.W. 57-323, 6-mercaptopurine riboside, and 2-amino-6-benzylthiopurine. The S-substituted 6-thiopurines and the 9-alkyl-6-thiopurines were scored as inactive, as were 8-azaguanine, 8-azaadenine, and 8-azadiaminopurine. Pyrimidine antagonists, such as 5-fluorouracil and 6-azathymine, as well as the antifolic acids, pyrimethamine and B.W. 50-197, also were scored as inactive.

Orbach-Arbouys and Eyquem (254), using human erythrocytes in chicks, found 6-MP, chlorambucil and actinomycin D active, but treatment with busulfan and thiotepa resulted in titers higher than those of the controls.

Santos *et al.* (291) employed sheep erythrocytes in the rat. Cytoxan, 6-mercaptopurine riboside, MTX, and B.W. 57-323 were selected as strongly active, 6-MP as possibly less active, while 5-fluorouracil and its deoxyriboside, vinblastine, and HN2 caused only minimal effects. The same authors have studied the effects of some of these drugs on the production of antibodies in man (patients with lymphomas or leukemia) to a purified Vi antigen and *Pasteurella tularense* vaccine (292). With the Vi antigen the antibody response could be completely suppressed by 7 days' treatment with cyclophosphamide, 6-MP, or 5-fluorodeoxyuridine, but HN2 had no effect. Nitrogen mustard, given 4 hours before the *tularense* antigen, also failed to suppress the subsequent rise in antibody, but 5-fluorouracil was active in 6 of 7 patients. None of these treatments affected pre-existing sensitivity reactions.

Humphreys *et al.* (176) have adapted their studies of the homograft rejection of L1210 leukemia to the screening of drugs for their ability to suppress this rejection. This test is limited, however, to pretreatment with drugs (except where drug-resistant lines of the leukemia are available) and it is not surprising that only the alkylating agents and X-irradiation exhibited marked activity; 6-MP, however, was moderately active, but MTX was relatively inactive.

Dutton and Pearce (91) compared the effects of a number of drugs in isolated spleen cell preparations, taken from animals that had been immunized to two different antigens, with a 1-day interval between antigenic stimuli. The cells, which were taken 2 days after the second antigen, were thus in two phases of antibody synthesis, and a differential effect on "2-day" as compared with "3-day" synthesis was believed to reveal selective effects on the development of antibody-forming capacity, rather than on synthesis *per se*. The thymidine analogs, 5-bromodeoxyuridine and 5-iododeoxyuridine, as well as 6-azauridine and possibly 6-MP, showed such differential effects, while ethidium bromide, thioguanosine, mitomycin C, and 5,6-dichlorobenzimidazole riboside failed to do so (91). Although the authors' interest appears to have been directed primarily toward the inductive period, it may be pointed out that this period was nearing completion when the experiments were carried out.

Butler (48) tested the effects of drugs, including a number known to suppress the primary response, on the secondary response in mice to diphtheria toxoid. Slight depression was seen with 6-MP, actinomycin D, and 4-nitroquinoline-N-oxide, but alkylating agents, MTX, hydrocortisone, and chloramphenicol were inactive. Although the doses which were selected for many of the drugs appear to have been reasonable, that for 6-MP (4.0 mg/kg) was so grossly inadequate that the detection of any effect at all is surprising.

In a broad way the screening tests have selected the same or similar compounds as inhibitors of the immune response. There is general agreement that alkylating agents, like radiation, are most effective when given before the antigen and that antimetabolites are most effective when given during the induction period. When one comes to details, some differences between drug effects, as related to species, antigen, timing and so on are discernible, but it would be premature to attempt to sort these out in view of the paucity of side-by-side comparisons.

F. Inflammation

Inflammation resembles sensitivity reactions in the migration of lymphocytes into the affected site, but is distinguishable from the latter by the near absence of an induction period. Many substances, including corticosteroids (*e.g.*, 100), are believed to suppress the inflammatory response. Similar effects are shown by some of the antimetabolites which inhibit the immune response. Strel'nikov found cytosine and isocytosine as effective as the standard phenylbutazone (329) and 2,6-dimercapto-5-amino-4-methylpyrimidine somewhat more effective. Page *et al.* studied the anti-inflammatory effects of 6-MP in detail. Pretreatment of rabbits with this drug for a period of 9 to 14 days markedly reduced the subsequent responses to a subcutaneous injection of egg-white (258, 260). The effects were obtained with minimal toxicity; in any case, animals in which the white blood cell counts had been reduced by treatment with HN2 responded normally, in contrast to those treated with 6-MP. Adrenalectomy did not influence the response to treatment with 6-MP. Histological studies showed that neutrophils appeared at the site of injection on schedule, but the normal migration of lymphocytes did not occur. Three possible interpretations were suggested; these involved 1) blockade of an informational process in the macrophage [*cf.* Fishman (114)], so that substances chemotactic for lymphocytes are not produced, 2) blockade of the responding mechanism in the lymphocyte, and 3) injury to or destruction of the small fraction of the total mononuclear cell population which responds. The requirement for prolonged pretreatment favors the last hypothesis, when one takes into account the rapidity with which 6-MP affects the induction of circulating antibodies.

Page *et al.* (259) have also demonstrated an anti-inflammatory effect of 6-MP in patients at levels (1.5 mg/kg) which did not depress the white blood cell counts, and have found cortisone to be relatively less effective. The response of patients with plasma cell hepatitis to 6-MP resembles the anti-inflammatory action of the drug in its temporal relationship, *i.e.*, improvement begins 2 to 3 weeks after the onset of therapy (259). The delay of several days to as long as

30 days in the response of leukemia patients to therapy with 6-MP should be called to mind.

IV. TREATMENT OF AUTOIMMUNE DISEASE

A. Introduction

The very mention of "autoimmune disease" in man brings forth screams of horror from the purists among the immunologists, who insist that no such entity has been established. One has a modicum of sympathy with this viewpoint, since there is in fact no clinical entity which will satisfy all the criteria (such as Milgrom and Witebsky's 5 points[4]) of a rigidly defined autoimmune disease. Nevertheless, there is a large body of human disease of mysterious origin, in which precipitating antibodies of some sort have been demonstrated. One can argue that the antigens are not autologous, but homologous or heterologous, or that they are autoantigens so modified by injury or disease that they escape recognition as self. In many instances it is not clear which came first, the disease or the antibody. Moreover, the antibodies might have been formed against spurious autoantigens or only against homoantigens and might lack sufficient discrimination to prevent reaction against self; indeed, it has been argued that the autoantibodies are not real antibodies but abnormal γ-globulins formed adventitiously and not in response to antigenic stimulus. One or more of these viewpoints may eventually receive such cogent support that it will have to be accepted. The purist may argue that the burden of proof lies with those who wish to use the term "autoimmune disease" and this is a valid argument from the standpoint of logic. Nevertheless, the term is well established in medical practice and will continue to be used, however loosely and poorly defined. In the broad, vague, and somewhat objectionable sense of a disease in which there is some evidence for the presence of antibodies reacting against tissues or organs, it serves to bring together for discussion under one heading a variety of diseases which show more or less clearly defined responses to "anti-immune" drugs. For discussions of the fine points the reader is referred to the excellent reviews of the field (45, 46, 79, 238, 272, 366).

B. Experimental autoimmune disease

1. Experimental allergic encephalomyelitis (EAE) can be produced in a variety of species (mice, rats, guinea pigs, rabbits, and monkeys) through the injection of brain or spinal cord which has been emulsified with a Freund's adjuvant. Within a short time neurological disturbances develop, as the result of demyelinating processes (2, 61, 370), and paralysis and death ensue. The relative roles of circulating antibodies and delayed hypersensitivity, however, remain uncertain.

Treatment with ACTH or cortisone (107, 109, 188, 242), salicylates (109, 136), radiation (63), or alkylating agents (7, 169, 188) produced some measure of

[4] Milgrom and Witebsky (238) suggested that in a completely satisfactorily defined autoimmune disease it would be possible to demonstrate 1) circulating antibodies, 2) a specific antigen, 3) the production of antibodies to this antigen in animals, 4) pathological changes attributable to the antibody, and 5) passive or adoptive transfer of the disease.

control of EAE, but none of these regimens was effective if started after the antigen was administered. Mercaptopurine protected animals which had not yet developed paralysis (136, 167, 169), but failed to cause remissions in those already paralyzed (170). Pretreatment and short courses of treatment were ineffective. Dosage was highly important; greatly enhanced effects were obtained with a relatively small increase in dosage (170). The important difference between 6-MP and other drugs was that treatment with it was effective when delayed until close to the time of appearance of symptoms. However, when therapy was discontinued, the disease appeared after a latent period, which was not modified by the duration of the treatment.

Several workers have reported that 6-MP is ineffective in combating EAE in guinea pigs (109, 346), but this failure is clearly to be attributed to inadequate dosage: 5 or 6 mg/kg per day contrasted with the 50 mg/kg intramuscularly and 150 mg/kg intraperitoneally used by Hoyer *et al.* (170). The last-named group could delay treatment in guinea pigs as well as in rabbits and still get suppression. Therapy with 6-MP is thus contrasted with radiation, which is effective in rabbits but not guinea pigs (63, 108).

2. Experimental immune thyroiditis has been produced in rabbits, guinea pigs, and dogs by the intradermal injection of either thyroid extract or thyroglobulin which has been emulsified with Freund's adjuvant (282, 367–369). Generally, considerable species specificity as regards the source of antigen is shown. The experimental animals develop a thyroiditis and circulating antibodies to thyroglobulin. Spiegelberg and Miescher (315) have studied the effects of 6-MP in guinea pigs which were immunized with two intradermal injections of thyroglobulin emulsion given 5 days apart. The treated animals (75 and 150 mg/kg per day, begun with the first antigenic stimulus) showed a greatly reduced incidence of thyroiditis. The effect of treatment on hypersensitivity was demonstrable but not striking, and all animals formed circulating antibody to thyroglobulin in titers comparable with those of the controls. In a second experiment, treatment was limited to a 10-day course beginning either at the time of the first antigenic stimulus or on the tenth day. Both groups showed effects similar to those observed in the first experiment.

C. Clinical autoimmune disease

1. Idiopathic thrombocytopenic purpura is characterized by erratic platelet counts, which at times reach extremely low levels. Evidence for circulating antibodies against megakaryocytes (224) and platelets (92) has been put forward. Splenectomy is stated to be effective in selected cases (160), and corticosteroids give good remissions in some patients (92). Rundles (288) has reported an interesting result of therapy with B.W. 57–323 in one patient whose disease had previously been controlled with difficulty with prednisolone. The drug at first appeared to be depressing the low platelet count even further, but when therapy was stopped there was a striking rebound of platelet count to normal levels. Eventually a regimen of 3 days' therapy per fortnight was worked out and this succeeded in maintaining a low normal thrombocyte count. Later a similar

regimen with azathioprine was used; this resulted in fewer side-effects and possibly better control of the platelet count. Some "striking responses" have been reported by Dameshek (75) using 6-MP and TG.

2. Autoimmune hemolytic anemia is characterized by a rapid destruction of erythrocytes, with resulting low red cell counts and hemoglobin levels, despite active blood regeneration as evidenced by high reticulocyte counts. Erythrocytes from such patients are positive by the Coombs test; from such cells antibodies can be eluted which react with normal as well as the patient's own reticulocytes (76, 238). Corticosteroids are beneficial in the majority of such cases, but sometimes have to be given at maximum tolerated doses to maintain control, and their use eventually may have to be interrupted or terminated because of the side effects of the steroids. The thiopurines appear to provide alternate therapy (75, 300). Thus, of 9 patients who had failed to respond to corticosteroid therapy, 4 gave good responses to either 6-MP or TG, while 5 previously untreated patients all responded (300). Similar results have been obtained with azathioprine (287).

3. Glomerulonephritis may exhibit antihuman kidney antibodies (189, 204) and thus may resemble the experimental nephrotoxic nephritis of rabbits (15, 185). Nitrogen mustard has been reported to have beneficial effects (186, 271), but the response has been highly variable (9, 54). Combined treatment with prednisone and azathioprine had no effect other than a possible slowing of the downhill course in 3 patients with glomerulonephritis. One patient with advanced renal failure and disseminated lupus showed marked clinical improvement with reversal of the L.E. preparation and return of the Coombs test to negative, but a second showed only an equivocal response (232).

4. Rheumatoid arthritis is accompanied by serological changes of an immunological character (99, 358) which are manifold but poorly understood. A rheumatoid factor, which combines with the patient's own γ-globulin, may participate in the development of the pathological condition (231b, 238), but the disease may occur in patients with agammaglobulinemia (135). Corticosteroids have been found to give striking remissions of the disease and to cause a reversion of some of the abnormal serological features to normal (157, 273, 359). Corticosteroids and salicylates have played the major roles in the management of this disease. Some rather gingerly explorations of the effects of antimetabolites have been undertaken within recent years. Aminopterin appears to diminish the signs and symptoms without a significant change in the laboratory values (138). One rather striking effect of an 18-day course of therapy with 6-MP has been reported (345).

5. Lupus erythematosus is classed as an autoimmune disease on the basis of the demonstration of circulating antibodies which react with deoxyribonucleoproteins (10, 163, 164, 181, 325) and denatured deoxyribonucleic acid (326). Steroids (164) and the 4-aminoquinoline antimalarials (13, 87, 164, 234) are used clinically with some success. The disease sometimes responds dramatically to HN2 (255). Exploratory studies with thiopurine derivatives have been reported. Thus Dameshek found at times striking effects on the disease, but a high

incidence of hematologic and gastrointestinal side effects (75, 78). This author suggested the possibility that a regimen of 6-MP followed by corticosteroids might be useful (75). Eisen *et al.* (96) found TG to give good responses in 2 patients, but one of these had a fatal gastrointestinal hemorrhage. Merrill (232) reported marked clinical improvement in one patient with disseminated lupus on treatment with prednisone and azathioprine, but a questionable response in a second. Lee *et al.* (199) reported resolution of the symptoms in a case of systemic lupus treated with 6-MP at a level which was discontinued because of toxicity. However, the patient improved rapidly on subsequent treatment with hydrocortisone.

6. Psoriasis exhibits no clear-cut evidence of an autoimmune component and the lymphocytic infiltrations which underlie the skin lesions ordinarily would be interpreted as an inflammatory reaction. It may be useful to mention the therapeutic effects of the antimetabolites at this point, because in many ways the responses to these in psoriasis are similar to those in autoimmune diseases. Many cases of psoriasis are manageable with only symptomatic treatment, but a considerable number are intractable enough to encourage experimentation with more drastic treatment. Corticosteroids alone have little effect but may act synergistically with the antifolic acids (72). The latter have produced improvement in a substantial percentage of the total number treated (251, 276, 328); the effectiveness as well as the toxicity is reversed by folic acid (72). Although toxic side effects occurred with considerable frequency in the earlier work (277), management without undue difficulty has been achieved with greater experience (276). Rees and Bennett (277) reported pyrimethamine inactive in a dose of 25 mg/kg for 1 month. However, this dose was without toxicity and may have been insufficient to produce a folic acid deficiency. The effects of 6-MP appear to be comparable to those obtained with aminopterin and amethopterin, although Rees and Bennett found that a 6-day course was without effect. Kravetz and Balsam (190) reported good to excellent responses in 16 of 24 courses of therapy (which averaged 32 days in length) in 12 patients. It is of some incidental interest that the antimalarials which are useful in lupus generally exacerbate psoriasis (13, 70).

7. Miscellaneous "autoimmune" diseases. Reports have appeared of the clinical effects of thiopurines in a variety of rare diseases of putative "autoimmune" character. These are shown in tabular form below:

Diagnosis	Therapy	Result	No. of Cases	Reference
Erythema nodosum	Azathioprine	Excellent	1	288
Periarteritis nodosa	Azathioprine	No effect	1	232
Plasma cell hepatitis (see above, Section IIIF)	6-MP	Control	3	259
Idiopathic plasmacytosis	TG	Partial	1	96
Atopic dermatitis	TG	Good	2	96
Systemic scleroderma	TG	Improved	1	96

For a considerable number of diseases there exists more or less cogent evidence of an immune component. These disorders appear to be prime targets for future investigations with antimetabolites which possess "anti-immune" effects. They include Hashimoto's thyroiditis (201, 231a, 256, 281, 367–369); myasthenia gravis (22); various liver diseases (130, 267); multiple sclerosis (2, 239); heart diseases (81); ulcerative colitis (42); sympathetic ophthalmia (79); and lung diseases (334). Several reviews discuss the evidence for the participation of immune reactions in a number of diseases (55, 79, 201, 238, 272, 366).

V. CONCLUSION

It is far too early to assess the ultimate role of the antimetabolites in the therapy of autoimmune diseases in general. In a few specific instances, such as the thiopurines in autoimmune hemolytic anemia and the antifolic acids in psoriasis, these uses are approaching the state of recognized therapeutic regimens. However, as several authors have pointed out, the drugs presently available provide little margin between the dosages required for therapeutic effects and those which will produce serious toxicity. Very little is available in the way of comparative effects of several antimetabolites in a given disease. Moreover, on the basis of present results, one is unable to sort out in a given situation the relative importance of effects on circulating antibodies, delayed hypersensitivity, and anti-inflammatory activities, although there are some indications that these effects may be at least partially separable.

One may reasonably inquire, in view of their less marked effects on established immunity than on the induction of the primary response, why these drugs influence autoimmune disease at all. Perhaps another apparent anomaly might be mentioned. The induction of tolerance requires a high initial dose of antigen, and tolerance is maintained only in the continuing presence of antigen. The persistence of a simple antigen is significantly increased when a carrier, such as Freund's adjuvant, is given simultaneously. One might expect that by this means the induction and maintenance of tolerance to the antigen would be facilitated, but the opposite is true. Whether this is the result of the route or site of administration or the nature of the carrier, it appears that the adjuvant may have altered the antigenic stimulus in some fundamental way. One clue which may provide future information is the observation of Mathé *et al.* (222) that different cell types are stimulated by different antigens and indeed by the same antigen with and without adjuvant. Similarly, Bieber (23) observed that the repressive effects of purine antagonists as opposed to those of thymidine affected different portions of the cellular response of the spleen to heterologous erythrocytes. Studies such as these eventually may aid in the classification of drugs by their effects on specific antigenic stimuli and may aid in the understanding of some of the species differences in responsiveness and in the interpretation of the responses obtained in the autoimmune diseases.

The mechanism of action of the antimetabolites in their effects on the immune response cannot be defined with any certainty at this time. Since most of the drugs which inhibit immune processes are also "anticancer" agents, a mecha-

nism involving cellular destruction first comes to mind. It can be shown that effects on immunity can be obtained at levels of drug which produce no obvious effects on the numbers of circulating lymphocytes, but this does not exclude the possibility of a selective destruction of a small clone of cells in which the rate of multiplication has increased in response to an antigenic stimulus. An evaluation of these factors may be facilitated if new drugs become available which show a greater separation of "anti-immune" and "antitumor" effects (161). Alternative explanations might involve a metabolic block of some informational process through inhibition of the biosynthesis of a nucleic acid. This might take the form of an inhibition of the synthesis *de novo* of the purine and thymine moieties of the nucleic acids. In the case of the base analogs, a direct incorporation might occur into one or more of the nucleic acid fractions, either deoxyribonucleic acid, or informational, soluble, or microsomal ribonucleic acid. The principal hindrance to a direct attack on these problems is the present inability to obtain the cell line concerned with a specific immune response in anything like a pure culture. It is impossible to carry out meaningful biochemical studies on cell populations in which only a small percentage of the cells is participating in the reaction to be studied. This may be summed up by saying that there is no solid foundation for an interpretational structure, but that a multitude of challenging problems exists. It is predictable that the solutions to many of these will come with further progress in studies of the immune response *in vitro*, as further advances are made in achieving uniformity in pertinent cell populations.

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