Chemical Suppression of the Immune Response

INTRODUCTION

This symposium dealing with the "Chemical Suppression of the Immune Response" was held in New York on February 7, 1962, just prior to the Fifth Biennial Tissue Transplantation Conference. It was organized by Drs. William Dameshek and Robert S. Schwartz of the Tufts–New England Medical Center, with the financial assistance of the Burroughs–Wellcome Co. of Tuckahoe, N. Y. and the advice of Dr. George Hitchings of that company. The considerable interest in this field was apparent from the number of abstracts submitted and the large attendance at the all-day meeting. It is unfortunate that the full texts of the papers presented as well as the discussions could not have been recorded, but doubtless these will eventually appear in the literature, albeit in piecemeal fashion. Drs. Dameshek and Hitchings alternated as Chairmen of the two sessions.

THE EFFECT OF CERTAIN ALKYLATING AGENTS AND ANTIMETABOLITES ON THE PRIMARY AGGLUTININ RESPONSE OF RATS INJECTED WITH SHEEP ERYTHROCYTES

G. W. Santos and A. H. Owens, Jr., Johns Hopkins University, School of Medicine, Baltimore, Md.

This presentation represents a preliminary report of the comparative effects of various agents on the primary immune response in the rat. Sprague-Dawley female rats, 10–12 weeks of age, were used as test animals. Nitrogen mustard, Cytoxan, 6-MP, 6-MP Riboside, ITG [2 amino-6(1' methyl-4'-nitro 5'-imidazolyl) thiopurine], 5 FU, 5 FUDR, methotrexate (MTX), and vincaleukoblastine (VLB), were injected i.p. daily (HN2 was given i.v.) for 5 days at doses 20 to 50 per cent of the LD50. One ml. of sheep erythrocytes (1 per cent) was injected i.p. at 4 hours and 48 hours after beginning of treatment, and at 48 hours before treatment. Animals were bled and titered individually for sheep cell agglutination at 4, 7, 10, 14, 21, 28, 35, and 42 days after injection of sheep cells. The data were analyzed for induction time, peak titer and average titer. All of the agents employed severely depressed peripheral blood counts and caused marked weight loss. VLB, HN2, 5 FU and 5 FUDR increased the induction period slightly over that of controls, but otherwise were inactive. Cytoxan, 6-MP Riboside, ITG, and MTX had relatively profound effects on all three of the parameters analyzed. Thirty to 100 per cent of the animals treated with the latter agents showed no agglutination for 42 days. The effect of 6-MP was intermediate to those of the aforementioned compounds. Although statistically significant only in the case of methotrexate, the data for all the "active" compounds suggests that there is greater suppression of antibody production when the antigen is injected 48 hours before treatment as compared to 4 or 48 hours after treatment. The results will be discussed briefly in terms of the clonal selection theory and mechanism of drug action.

SOME ASPECTS OF THE RESPONSE OF MICE TO THE ADMINISTRATION OF SHEEP RED BLOOD CELLS

Samuel Bieber, Henry C. Nathan and Dona C. Hooper, Burroughs Wellcome & Co., Wellcome Research Laboratories, Tuckahoe, N. Y.

The need for screening tests for the detection of agents which interfere with the immune response prompted the development of a method utilizing the agglutinin response in mice to the administration of sheep red blood cells (Nathan et al.: Proc. Exper. Biol. & Med. 107:796, 1961). Two aspects of this phenomenon have been studied: the disposition of Cr51-labeled sheep red blood cells and the morphologic changes in liver and spleen. A modest decrease in Cr51-levels in the peripheral circulation is noted.
during the first 48 hours after the administration of Cr51-labeled sheep cells to mice. This is followed by rapid clearance to completion by days 5–6. Cr51-levels in liver and spleen reach a peak at day 3, and diminish thereafter, but are detectable 7 days after clearance from the peripheral circulation is complete.

The liver parenchyma shows evidence of diffuse damage at days 2–4, followed by reparation. Kupffer cell activity is detectable 24 hours after antigen administration. Mitotic activity reaches a peak by day 6 and is associated with a streaming of these cells towards the sinuses. This period is also marked by a significant accumulation of lymphocyte-like cells. Alterations in the histomorphology of the spleen are more dramatic. The germinal centers show marked activation within 24–48 hours with the appearance of large numbers of pyroninophilic, mononuclear cells as well as giant cells. This activity reaches a maximum by days 6–7 and is associated with a reduction in the red pulp. Drugs which have been demonstrated capable of suppressing the appearance of hemagglutinins were found to modify the clearance of Cr51-labeled antigen and to suppress many of the responses of the liver and spleen.

**The Effect of Methotrexate on Antibody Production in the Dog**

E. D. Thomas, J. A. Baker, D. A. Blumenstock, H. Hechtman and J. W. Ferrebee,
Mary Imogene Basset Hospital, Cooperstown, N. Y.

The effect of methotrexate on antibody production in the dog has been assessed by measuring the response to an attenuated distemper virus vaccine. Normally, antibody production begins in 6 to 8 days and reaches peak levels in 21 to 28 days. In dogs given methotrexate in doses of as little as 0.1 mg. per Kg. three times per week, antibody production is completely suppressed for 3 weeks. If the drug is discontinued at that time, antibody production begins in about 5 days and proceeds at optimal rates. If the drug is continued, antibody production occurs at a greatly reduced rate.

About half the dogs vaccinated while on methotrexate die at the end of three weeks whereas control dogs on drug alone show no sign of toxicity. The dogs dying at this time show no antibody production and vaccine virus can be isolated from their blood. The production of a fatal illness by the usually benign vaccine virus may explain some of the difficulties with “methotrexate toxicity” in experimental animals subjected to irradiation and homografting procedures. That methotrexate also has a profound effect on transplantation immunity in the dog is indicated by a prolongation of skin graft survival, lung graft survival and a decreased incidence of secondary disease in irradiated dogs with marrow homografts.

**Prevention of Secondary Immune Response with 6-Mercaptopurine**

E. S. LaPlante, R. M. Condie and R. A. Good, Pediatric Research Laboratories of Variety Club Heart Hospital, University of Minnesota, Minn.

6-Mercaptopurine was known to inhibit the primary immune response in rabbits when given in a dose of 6 mg./Kg./day and to have negligible effects on the secondary immune response. In the present study, a 25-day course of 6-MP, in this dosage, before secondary stimulation, produced significant depression of antibody production to bovine serum albumin, but did not prevent the response. When the drug was given in higher doses, 12 and 15 mg. per Kg., beginning at the time of secondary antigenic stimulation, the secondary response was regularly inhibited, with no evidence of undue toxicity from 6-MP. The findings suggest that prior failure to prevent the secondary immune response with 6-MP resulted from inadequate doses or use of inactive preparations of the drug. In a pilot study with a small number of animals, it was suggested that 6-MP also interferes with the tertiary immune response. These animals had received 6-MP at the time of secondary stimulation with BSA and had shown no antibody. The tertiary response was also deficient, although no additional
6-MP was given. Thus, 6-MP differs from such agents as total body irradiation and cortisone in suppressing at least the primary and secondary responses, and perhaps interfering with immunologic memory as well.

**DELAYED TREATMENT OF IMMUNE PROCESSES WITH 6-MERCAPTOPURINE**

**R. M. Condie and N. R. Forscn**, Pediatric Research Laboratories of Variety Club Heart Hospital, University of Minnesota, Minn.

The finding that steroids, nitrogen mustard, and total body irradiation failed to either prevent the secondary immune response or to interfere with the primary response when administered hours after antigenic stimulation has determined the treatment schedules that have been used in studies on the chemical suppression of immunity. In studies on the effect of various antimetabolites on the immune response, delayed treatment (treatment started after antigenic stimulation) with these compounds has been neglected. The suppression with 6-mercaptopurine (6-MP) of the secondary response suggested that this purine analogue might interfere directly with antibody production and that delayed treatment should interfere with both primary, secondary, and hyper-immune processes. In the primary response, 6-MP administration delayed up to the 7th day following antigenic stimulation prevented the appearance of circulating antibodies. The secondary immune response could be completely prevented by treatment delayed one day after antigenic stimulation while 6-MP administration started 4 days following stimulation interfered with antibody production. Delayed treatment has not been effective in altering delayed hypersensitivity or the homograft rejection. However, experimental allergic encephalomyelitis (EAE) like antibody production was prevented with delayed treatment schedules started 9 days following antigenic stimulation. The implication of these findings on the pathogenesis of EAE on current concepts of the "induction period" of the immune response and on the clinical application of the delayed treatment concept will be discussed.

**ANAoLYSIS OF A TOLERANCE PHENOMENON EVOKED IN ADULT MICE BY CARCINOGENIC HYDROCARBONS**


Increased tolerance to the transplantation of normal and neoplastic tissues has been demonstrated in certain strains of mice following treatment with polycyclic hydrocarbons. This effect can be quantitated, and the level of tolerance produced is correlated with the degree of epidermal carcinogenicity of these compounds. Different strains of mice vary in susceptibility to this effect of carcinogens. The character of sensitivity is inherited as a single gene recessive and is independent of the known histocompatibility loci.

Tolerance can be demonstrated with homologous tumors which can be made to grow progressively—at the same rate and with the same outcome as in the strains of origin of the tumors. It is also possible to transplant skin successfully. The degree of success and the length of survival increases as the histocompatibility of the donor approaches that of the carcinogen-treated recipient. Evidence from several lines of experimentation all indicate that the mechanism of tolerance induction depends upon an active immune process rather than on interference with antibody production. It is perhaps similar to the "enhancement" phenomenon of Snell and Kaliss.

**DRUG-INDUCED IMMUNOLOGIC "TOLERANCE" FOR HOMOTRANSPLANTATION OF SKIN**

**Delta E. Uphoff and Louisa Pitkin**, National Cancer Institute, Bethesda, Md.

Following the experimental design previously reported (Trans. Bull. 28:110, 1961), pretreated mice were tested for "tolerance" by skin homografts. Male
Suppression of Delayed Hypersensitivity in Guinea Pigs and Rabbits with 6-Mercaptopurine

J. R. Hoyer, R. M. Condie and R. A. Good, Pediatric Research Laboratories of Variety Club Heart Hospital, University of Minnesota, Minn.

Guinea pigs and rabbits infected with BCG develop characteristic delayed (tuberculin) hypersensitivity (DH) to PPD. 6-Mercaptopurine (6-MP) i.m. (50 mg./Kg./day) suppressed the development of DH during the period of administration in guinea pigs. When 6-MP was started after development of delayed hypersensitivity, treatment failed to suppress DH. Starvation (accompanied by a 40 per cent weight loss) started at time of infection did not prevent development of DH. Lower doses and intraperitoneal injections of 6-MP were less effective in suppressing DH. Rabbits infected with BCG were treated with 6-MP in doses from 6-18 mg./Kg./day i.v. Six to 18 mg. of 6-MP suppressed DH when started at time of infection but not after DH had developed. The marked suppression of DH with 6-MP is dependent on route, timing, and dosage of 6-MP. Guinea pigs required more 6-MP to suppress DH than did rabbits.
Recovery of Guinea Pigs from Primary Vaccinia Virus Infection Following Administration of Methotrexate to Suppress Immune Responses

Robert M. Friedman, Laboratory of Pathologic Anatomy, NCI, Samuel Baron and Charles Buckler, LBV, NIAID

It has been shown that guinea pigs whose antibody response had been blocked by radiation recovered from vaccinia virus infection as rapidly as did normal animals. The irradiated animals, however, developed delayed skin hypersensitivity to vaccinia virus. When methotrexate was administered to suppress the development of the delayed hypersensitive reaction, it became possible to study the recovery of guinea pigs from vaccinia virus infection in the absence of delayed hypersensitivity.

Administration of 5 mg. of methotrexate every 48 hours to animals previously x-irradiated and then infected with vaccinia virus suppressed both antivaccinial antibody production and the development of delayed hypersensitivity to vaccinia virus. The irradiated animals which received the methotrexate were free of lesions and eliminated virus as soon as nonirradiated animals without methotrexate treatment. These findings are consistent with the hypothesis that the immune response may not be essential for recovery from a primary vaccinia virus infection and that less specific mechanisms, such as interferon production, may be more closely associated with the recovery process than antibody.

The Effect of 6-Mercaptopurine on Experimental Allergic Encephalomyelitis

L. W. Hoyer, R. M. Condie and R. A. Good, Pediatric Research Laboratories of Variety Club Heart Hospital, University of Minnesota, Minn.

Clinical and histologic evidences of experimental allergic encephalomyelitis (EAE) in rabbits have been completely suppressed by the daily intravenous administration of 12 mg./Kg./day of 6-mercaptopurine (6-MP). This suppression continues as long as the drug is administered, but when treatment is discontinued the disease develops after a latent period of 9 days. Suppression of the disease in rabbits can also be achieved when treatment is begun up to 12 days after the injection of the spinal cord-adjuvant emulsion. After 3 days of treatment, no further paralysis is observed, but no evidence of remission was observed in animals already paralyzed. We have found that 6-MP has a similar effect in suppressing EAE in the guinea pig. Higher dosages are required in this species, but by using 50 mg./Kg./day intramuscularly we can suppress the disease with immediate or delayed treatment.

Because 6-MP did not produce severe debility or leukopenia in the dosages used, the drug's action has been considered to be an interference with the immunologic response to the spinal cord-adjuvant which is essential in the pathogenesis of this disease. 6-MP provides the best control over EAE of any of the agents or chemicals used in its treatment. It is the only way in which the disease can be 100 per cent prevented during drug administration, and it is the only drug which affects the disease when initial treatment is delayed.

Lymphatic Tissues and the Suppression of the Immune Mechanism

C. C. Congdon, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

One potential way of assaying for agents that suppress the immune mechanism is to study the effect of suppressor agents on the cellular changes that take place in the lymphatic tissues during foreign tissue or antigen rejection. A characteristic series of changes involving the spleen white pulp and lymph node cortex occurs during an immunizing process. Radiation markedly reduces or suppresses this series of changes...
depending on the quality of the antigenic stimulus. Reports from the literature indicate that cortisone has relatively little effect on the lymphatic tissue changes after foreign skin grafting, but that 6-mercaptopurine may markedly interfere with the lymph node response to skin homografts.

COMPARATIVE ANTITUMOR AND ANTI-IMMUNE EFFECTS


The screening of compounds for possible anti-immune effects, using the formation of sheep cell hemagglutinins in mice, has revealed a number of active substances (Nathan et al.: Proc. Soc. Exper. Biol. & Med. 107:796, 1961). The parallelism between anti-immune and antitumor activities (against adenocarcinoma 755) found in early tests does not seem to be universally true. Thus bromodeoxyuridine (BUDR) and thiodeoxyuridine (TUDR) show only minor effects in antitumor trials and major effects in the anti-immune testing, and closely-related analogues with equal or greater antimicrobial effects show only minimal inhibition of hemagglutinin formation. Similarly 6-

methylaminopurine and 6-benzylaminopurine are inactive against adenocarcinoma 755, but are reproducibly active as inhibitors of antibody formation. The 6-NIP. Combined treatment with BUDR and 6-MP results in about a 3-fold potentiation, and at maximum tolerated doses in greater suppression of antibody formation than can be obtained with either compound singly. Combinations of TUDR and 6-MP similarly show greater than additive effects. The possible significance of these results in terms of drug-suppression of the immune response will be discussed.

EFFECT OF ANTITUMOR AGENTS ON THE HOMOGRAFT RESPONSE

Abraham Goldisi, Stewart R. Humphreys and John P. Glynn, National Cancer Institutes, Bethesda, Md.

Studies in this laboratory demonstrated that immunity to L1210 leukemia developed in compatible (semi-isologous) F₁ hybrids during therapy of the systemic disease with halogenated derivatives of amethopterin. This observation seemed to conflict with reports that folic acid antagonists may interfere with the homograft reaction in homologous strains of mice. Because of this apparently contradictory nature of the mode of action of antifolics, additional studies were undertaken in homologous animals on sensitive L1210 leukemia and several antifolic-resistant sublines.

The studies showed that the homograft response to antifolic resistant sublines was overcome by treatment with amethopterin or dichloroamethopterin. However, the folic acid antagonists did not interfere as extensively with the induction of host immunity, and once immunity was induced, it was not as readily suppressed by treatment with folic acid antagonists. In extension of these studies, a system involving pretreatment of the host has been employed to test drugs for their ability to suppress the homograft response to transplantation of tumor and skin graft. Data will be presented on the effects of antifolies, alkylating agents, purine antagonists, etc. in this system.

THE EFFECT OF B.W. 57-322, 6-METHYLMERCAPTOPURINE, URETHANE PLUS 6-AZAURACIL, AND PREDNISOLONE ON RENAL HOMOGRAFT SURVIVAL

C. F. Zukoski and J. W. Callaway, V. A. Hospital and Vanderbilt Univ. School of Medicine, Nashville, Tenn.

In an attempt to prolong renal homograft survival in the dog, the above medications were administered orally the day of operation and daily thereafter to mongrel dogs which had bilateral nephrectomies and a renal homograft transplanted into the
pelvis. The average survival of non-treated dogs which had the same operation is 15 days. Of seven dogs given B.W. 57-322, one dog lived 148 days with an NPN of 49 mg. per cent. The cause of death was not found at post-mortem. Six-methylmercaptopurine was administered to 6 dogs. At present, one is alive at 157 days with an NPN of 49 mg. per cent, gaining weight after an initial period of weight loss associated with an elevated NPN. Urethane plus 6-azauracil is now being studied with two dogs alive at 21 days. Prednisolone alone (30 mg./day, orally) has been given to six dogs. One is now alive at 117 days with an NPN of 48 mg. per cent. The antimetabolites studied have shown a significant inhibition of antibody formation to purified protein antigens. B.W. 57-322, 6-methylmercaptopurine and prednisolone were found to be agents which allowed a very significant prolongation of renal homograft survival.

**Additive Inhibitory Effect of Urethane and X-Radiation on the Homograft Reaction in Mice and Dogs**

*Leonard J. Cole and William E. Davis, Jr., Division of Biological and Medical Sciences, U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.*

We have observed recently that combined treatment of mice with urethane plus sublethal x-radiation (500 rad) inhibits the homograft response to a degree considerably greater than that with x-irradiation alone. Adult (C57L x A)F1 mice received two injections of urethane (1 mg. per Gm. body weight) given one day apart, plus 500 rad of x-rays. Tail skin grafts from C3H donors engrafted one day after the irradiation, were retained for periods up to 60 days, with a mean graft survival time of 40 days, as compared with 18 days for mice exposed to 500 rad only. Combined treatment of LAF1 mice with urethane plus 6-mercaptopurine, followed by 500 rad, did not prolong the survival of C3H skin homografts over those on mice treated with urethane plus 500 rad. Urethane in conjunction with x-radiation has also been employed in studies on homologous marrow transplantation in dogs. In a typical experiment, mongrel male dogs were injected with urethane (175 or 350 mg./Kg.) for 3 or 4 days prior to whole-body exposure to 900 rad of 250-KVP x-rays (dose-rate = 17 rad/min.). One day after irradiation, the dog received a single intravenous injection of homologous bone marrow (approx. 10 x 10⁸ nucleated cells) from a mongrel female dog donor. Successful “take” of the donor marrow was observed, as indicated by recovery of the peripheral leukocyte count by 8 days postirradiation, and by the presence of female granulocytes in the circulating blood by the 11th day. Thus far, the dogs so treated have succumbed by the end of the third week postirradiation, with symptoms suggesting secondary disease. We have been unable to obtain increased survival time or successful “takes” of homologous bone marrow grafts in dogs receiving this supralethal dose of x-radiation without urethane. These results suggest that urethane potentiates the effect of x-rays in suppressing the homograft response in dogs, as well as in mice.

**Suppression of the Homologous Disease in Mouse Chimeras by Preirradiation of Donor Cells**

*Gustavo Cudkovicz, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.*

Bone marrow cells of (C57BL/6 × DBA/2)F₁ donor mice were suspended in newborn agamma calf serum and transplanted into lethally irradiated (C3H/Anf × 101)F₁ recipients. Early and delayed mortality of the chimeras was recorded thereafter, and at 90 days the peripheral blood hemoglobin was characterized to ascertain the presence of donor-type erythrocytes. The donor marrow was irradiated in vitro immediately before transplantation, with doses of x-rays ranging from 300 to 500 r; in some instances 3.2 mM of the radioprotective drug, S,β-aminoethylisothiouronium · Br · HBr (AET) were added to the marrow suspension 5-10 min. before
exposure to x-rays. Thirty-day survival of the recipient mice was dependent on the treatment given to the marrow cells; survival was insured when large numbers of irradiated cells were transplanted, up to $2 \times 10^8$ nucleated cells per mouse. For a given number of irradiated cells, however, the survival was greater when the marrow was irradiated in the presence of AET. Homologous disease among 30-day survivors was severe and frequent whenever unirradiated marrow was given. Irradiation of the donor cells markedly reduced the incidence of the disease to an extent which was dependent on the radiation dose and on the number of cells injected. Recipients of marrow irradiated in the presence of AET showed reduction in the incidence of homologous disease to the same extent as recipients of marrow irradiated in the absence of AET. Since the results are attributed to elimination of immunologically competent cells or precursors from donor marrow by x-irradiation, and since this effect was not lessened by AET, it is inferred that the immune cells were not protected against radiation injury to the same extent as other marrow elements, if at all.

**Bone Marrow Transplantation after Total Irradiation in Leukemic Mice Followed by Administration of a Chemotherapeutic Agent to Reduce the Secondary Syndrome and to Be Added to the Antileukemic Effect**

G. Mathe, J. L. Amiel and J. L. Niemetz, Centre de Recherches Cancérologiques et radiopathologiques de l'Association Claude-Bernard, and Hôpital Saint-Louis, Paris, France

$F_1$ (C57Bl6 x DBA2) mice were given $10^8$ leukemic leukocytes (L 1210) obtained from DBA2 mice. Total body irradiation (L.D. 100) followed by isologous bone marrow infusion very seldom eradicates the leukemic process or improves the survival rate. Total body irradiation followed by transplantation of bone marrow cells obtained from C57Bl6 mice cures the leukemic process in a fair number of animals. Many of these animals will die with secondary disease. In an attempt to dissociate these two entities, the antileukemic effect and the factors responsible for the secondary disease, animals were given two types of antimitotic agents (amethopterin and cyclophosphamide) after their bone marrow has recovered. The dosage was kept below what was believed to be the therapeutic level with the idea that these compounds could (a) enhance the antileukemic effects by reducing the mitotic rate of tumor cells, and (b) decrease the incidence of secondary disease by interfering with the division of immunologically competent cells from the bone marrow transplant. Preliminary results seem to confirm this hypothesis.

**Clinical Studies on the Anti-Inflammatory Activity of 6-Mercaptopurine**

A. R. Page, R. M. Condie and R. A. Good, Department of Pediatrics, University of Minnesota Medical School, Minneapolis, Minn.

We have now treated three plasma cell hepatitis patients with 6-mercaptopurine. Each of these patients has shown the same pattern of response with clinical and laboratory evidence of improvement starting 2 to 3 weeks after onset of therapy. In the one case who had no prior cortisone therapy, the liver function tests returned to normal in a few weeks although the extreme hypergammaglobulinemia has continued unaltered for more than 4 months. By study of the inflammatory response with the Rebuck skin window technic we have been able to demonstrate complete inhibition of the mononuclear cell response to inflammation in patients on a daily dose of 1.5 mg./kilo of 6-MP. Approximately 3 weeks of treatment is required before this anti-inflammatory effect can be demonstrated by the Rebuck technic. Further, this effect is seen in patients with normal white blood cell counts. Patients tested with cortisone in dosages of 300 mg./M2 had only moderate diminution of the cellular inflammatory exudate when studied by the Rebuck technic. It is probable that the marked
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clinical response in patients with chronic hepatitis is due to the anti-inflammatory effect of 6-MP rather than the suppression of antibody formation. (Aided by grants from the U. S. Public Health Service, Minnesota Heart Association, and the National Foundation.)

THE TREATMENT OF AUTOIMMUNE DISEASE WITH THE ANTIPURINE DRUGS

William Dameshek, Blood Research Laboratory, New England Center Hospital, Boston, Mass.

Because of two features, (1) the many analogies of autoimmunization with leukemia and (2) the striking effects of the antipurines on the immune response, these chemicals were used in the treatment of autoimmune disease. The results in autoimmune hemolytic anemia (AIHA), using the same doses as used in leukemia (2.5 mg./Kg./day) were usually very striking, and reactions were minimal. It was evident that these drugs could be used in place of the corticosteroids, when this seemed advisable, and that thus a new medical form of therapy was available. In systemic lupus, although the results were at times very striking, reactions to drug (gastrointestinal, hematologic) seemed much more common than in AIHA, thus curtailing drastically the continued use of the drug. On the other hand, it is possible that some of our very good results in systemic lupus were due to the prior use of 6-mercaptopurine followed by the corticosteroids. Therapy is difficult to evaluate in this very serious and very "tricky" disease. We have also been studying the effects of the antipurines in idiopathic thrombocytopenic purpura, particularly in the severe cases that are in relapse after all available therapy has been tried. Some of these cases have shown striking responses, but the possibility of injuring the bone marrow is always present. Thus, the use of the antipurines in the autoimmune diseases needs to be carefully assessed by further trials. These are potent drugs which often cause reactions. The newest analogue, B & W 57-3ww seems to be the best tolerated thus far. It is hoped that even better analogues will be synthesized.

TREATMENT OF RENAL DISEASE WITH COMBINED STEROID THERAPY AND ANTI-METABOLITES

John P. Merrill, Harvard Medical School, Boston, Mass.

Nine patients with renal disease have been treated with the combination of steroid therapy and BW-322 (a substituted 6-mercaptopurine): two patients with disseminated lupus erythematosus and renal involvement, three patients with membranous glomerulonephritis and the nephrotic syndrome, one patient with polyarteritis, and three patients with glomerulonephritis. BW-322 was given in doses varying from 2 to 7 mg. per kilo and in combination with prednisone, one mg. per kilo. No discernible effects were evident in the three patients with the nephrotic syndrome and membranous glomerulonephritis. In one patient with advanced renal failure and disseminated lupus, the L.E. preparation reversed, the positive Coombs' test became negative, and marked clinical improvement resulted. In the other patient with minimal renal involvement, the only evidence of beneficial effect was a flareup in symptoms when the antimetabolite was tapered. No striking change occurred in the patient with polyarteritis or those with glomerulonephritis although the clinical impression was gained that the rapid downhill course of the disease was slowed. The complications of therapy and the problems of evaluation will be discussed.
Chemical Suppression of the Immune Response