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Immunosuppression by Cytostatic Drugs?

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Summary

In the present study, an attempt was made to characterize the immunomodulating abilities of the cytostatic drugs cyclophosphamide, ifosfamide, vinblastine, vincristine, procarbazine, dacarbazine, 6-mercaptopurine, methotrexate, 5-fluor-uracil and adriamycine in a defined experimental model. Varying combinations of drug plus transplantation alloantigen, (C3H-lymphocytes) were injected into Balb/c mice at different time intervals in vivo. *The resulting T-effector cell reactivity was determined* in vitro *with the microcytotoxicity assay on day +5 for primary (1°) and day +7 for secondary (2°) sensitized mice. According to the type of drug (alkylating agent vs. vinca alkaloid vs. antimetabolite vs. cytostatic antibiotic), the dosage (20% LD_{50} vs. 60% LD_{50}), the state of sensitization (1° vs. 2° sensitized recipients), and the time of drug application in relation to the antigen treatment on day 0 (in varying steps from day −6 to day +4), so-called "pharmacon-antigen-variation-effects" (PAVE) were established for each of the investigated drugs in form of reaction profiles.*

The results were as follows: (1) For almost all substances, characteristic reaction profiles involving immunostimulation and/or immunosuppression could be established. Similarities in the profiles of different substances made it possible to classify the drugs according to different reaction types. The reaction type however is not definitely correlated to the biochemical mechanism of drug action. (2) The PAVE are decisively influenced by some of the biological parameters, such as the time of drug application in relation to the antigen treatment and the state of sensitization but relatively little by the dosage of the drug. (3) Considering the different processes occurring during primary and secondary immune responses, the PAVE may give hints for a distinct manipulation of the immunoregulation and thus information on the immunobiological mechanism of drug action.

Introduction

Classic cytostatic drugs as cyclophosphamide, azathioprine or methotrexate have generally been used as immunosuppressive agents in the treatment of autoimmune diseases, graft rejection reactions or in tumor chemotherapy. However, some years ago several authors (*Müller-Ruchholtz*, 1974; *Röllinghoff* et al., 1977) published results showing that under certain experimental conditions these drugs may not only cause immunosuppression but also immunostimulation; thus, these drugs could more appropriately be called "immunomodulators". Only very recently has the immunobiological mode of action of these drugs become the focus of more intensive research as was shown by the lively discussion on the new substance cyclosporin A (*Tutschka*, 1979; *Thomson*, 1983). Increasing interest in immunopharmacological questions however revealed the uncertainty in handling

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immunomodulating agents and the lack of knowledge about their underlying immunobiological mode(s) of action.

Single calls have been made for systematic experimental research (e.g. *Möller*, 1980) and *Hadden* et al. (1981) have particularly stressed the following problems: (1) In contrast to precise knowledge of the biochemical mechanism, the immunobiological mechanism of most of these substances is largely unknown. This deficit should be overcome, also in the interest of research on cell-mediated immunoregulation. (2) Animal experiments involving immunopharmacological agents are performed almost exclusively with primary sensitized organisms. The results of such experiments however are not transferable to the clinical situation, in which immunosuppressive treatment generally must presuppose a state of secondary sensitization. Therefore comparative studies of this essential biological parameter appear to be necessary. (3) For the increasing number of substances investigated a great number of unique and sometimes contradictory results are reported. Reasons for this are great differences in the mode of application of the drug, its dosage, the type and preparation of antigens used and last not least the test methods, all of which limit a direct comparison of the results.

Considering the present state of research, it becomes obvious how necessary systematic comparative experimental studies on drug manipulated immune reactivity are, which so far have only been presented by *Berenbaum* (1979) for the B-cell system. The present study tries to characterize and to compare the immunomodulating abilities of a variety of clinically used cytostatic drugs on the T-cell system in a defined experimental mouse model. Therefore so-called "pharmacon-antigen-variation-effects" (PAVE) were established for each of the investigated drugs in form of reaction profiles. The following questions were of particular interest: (1) How are the immunomodulating effects determined by the biochemical type of drug? (2) How are such effects influenced by the time and dosage of drug application and the state of sensitization of the recipient? (3) Do the results obtained from (1) and (2) elucidate the underlying immunobiological mechanism of drug action?

Material and Methods

Animals

The experiments were carried out with 2–3 month old inbred female Balb/c mice (H-2^d), weighing 22–30 g.

Sensitization

2–3 month old inbred female C3H mice (H-2^k) served as alloantigen donors. 5 x 10^7 spleen, thymus and lymph node cells, suspended in Hanks' balanced salt solution (HBSS) were injected i.p.. Primary (1°) sensitized mice received only one antigen injection on day 0, secondary (2°) sensitized mice two additional injections on day −17 and day −10.

Drugs

To allow for a direct comparison of the results, the LD_{50} for female Balb/c mice was determined for each drug separately according to the method of *Reed* and *Muench* (1938).

Cyclophosphamide, CY, (Endoxan, Asta-Werke, Bielefeld): 100 mg CY and 45 mg NaCl were dissolved according to the prescription. The injection solution was equilibrated with HBSS to 0.5 ml per 20 g body weight and injected i.p. within 30 min after preparation of the solution. 20% LD_{50} = 102 mg, and 60% LD_{50} = 306 mg per kg body weight (BW).

Ifosfamide, IF, (Holoxan, Asta-Werke, Bielefeld): 200 mg IF; for preparation cf. CY. 20% LD_{50} = 118 mg, and 60% LD_{50}= 354 mg/kg BW.

Vinblastine, VLB, (Velbe, E. Lilly GmbH, Gießen): 10 mg VLB sulphate; for preparation cf. CY. 20% LD_{50} = 1.5 mg, and 60% LD_{50} = 4.4 mg/kg BW.

Vincristine, VCR, (Lilly, E. Lilly GmbH, Gießen): 1 mg VCR sulphate and 10 mg lactose; for preparation cf. CY. 20% LD_{50} = 0.9 mg, and 60% LD_{50} = 2.6 mg/kg BW.

Procarbazine, PC, (Natulan, Hoffmann-La Roche, Basel): PC was dissolved in distilled water; for preparation cf. CY. 20% LD_{50} = 113 mg, and 60% LD_{50} = 339 mg/kg BW.

Dacarbazine, DTIC, (DTIC-Dome, Miles GmbH, Frankfurt): 200 mg DTIC, 200 mg citric acid and 75 mg mannit; for preparation cf. CY. 20% LD_{50} = 57 mg, and 60% LD_{50} = 171 mg/kg BW.

6-Mercaptopurine, 6-MP, (Puri-Netuol, Deutsche Wellcome, Burgwedel): 6-MP was dissolved in 0.12% NaOH; for preparation cf. CY. 20% LD_{50} = 33 mg, and 60% LD_{50} = 99 mg/kg BW.

Methotrexate, MTX, (Methotrexat, Lederle, München): 50 mg MTX and 4.8 mg NaCl; for preparation cf. CY. 20% LD_{50} = 38 mg, and 60% LD_{50} = 115 mg/kg BW.

5-Fluor-Uracil, FU, (Hoffmann-La Roche, Basel): 250 mg FU; for preparation cf. CY. 20% LD_{50} = 68 mg, and 60% LD_{50} = 204 mg/kg BW.

Adriamycine, AM, (Adriblastin, Farmitalia, Freiburg): 10 mg AM and 50 mg lactose; for preparation cf. CY. 20% LD_{50} = 4 mg, and 60% LD_{50} = 12 mg/kg BW.

Each Balb/c mouse received only one single drug injection (i.p.).

Combined treatment

To determine the immunomodulating abilities of the drugs, varying combinations of drug plus alloantigen were injected *in vivo* into Balb/c mice. The resulting "pharmacon-antigen-variation-effects" of the T-effector cells were determined *in vitro* according to the type of drug (alkylating agent vs. vinca alkaloid vs. antimetabolite vs. cytostatic antibiotic), the dosage (20% LD_{50} vs. 60% LD_{50}), the state of sensitization (1° vs. 2° sensitized recipients), and the time of drug application in relation to the alloantigen treatment on day 0 (in varying steps from day −6 to day +4). Test time was day +5 for 1° and day +7 for 2° sensitized mice (*Bröcker* et al., 1977). Mice receiving only antigen treatment served as controls.

Test procedure

For preparation of the target cell cultures and the T-effector cells and for the test procedure, cf. *Takasugi* and *Klein* 1970. Briefly, cytotoxic T-effector spleen cells from mice treated with drug and antigen, or only antigen were co-incubated with adherent target fibroblasts for 48 h in Terasaki microtest plates. The lysis of the target cells was read microscopically and calculated for each of the eight effector/target cell ratios (3:1 – 400:1) according to the following formula:

$$\text{specific lysis (\%)} = \left(1 - \frac{\text{remaining target cells (exp.)}}{\text{target cells (contr.)}}\right) \times 100.$$

Results

1. Primary sensitization

Figure 1 shows the "pharmacon-antigen-variation-effects" for the T-effector cell reactivity of 1° sensitized Balb/c mice which received a single dose of alloantigen on day 0. In addition they received a single dose of one of the cytostatic drugs varying from the 6th. day before to the 4th. day after the antigen. Two different dosages were applied, 20% and 60% of the LD_{50}. The T-effector cell reactivity of spleen lymphocytes was tested with the mic-

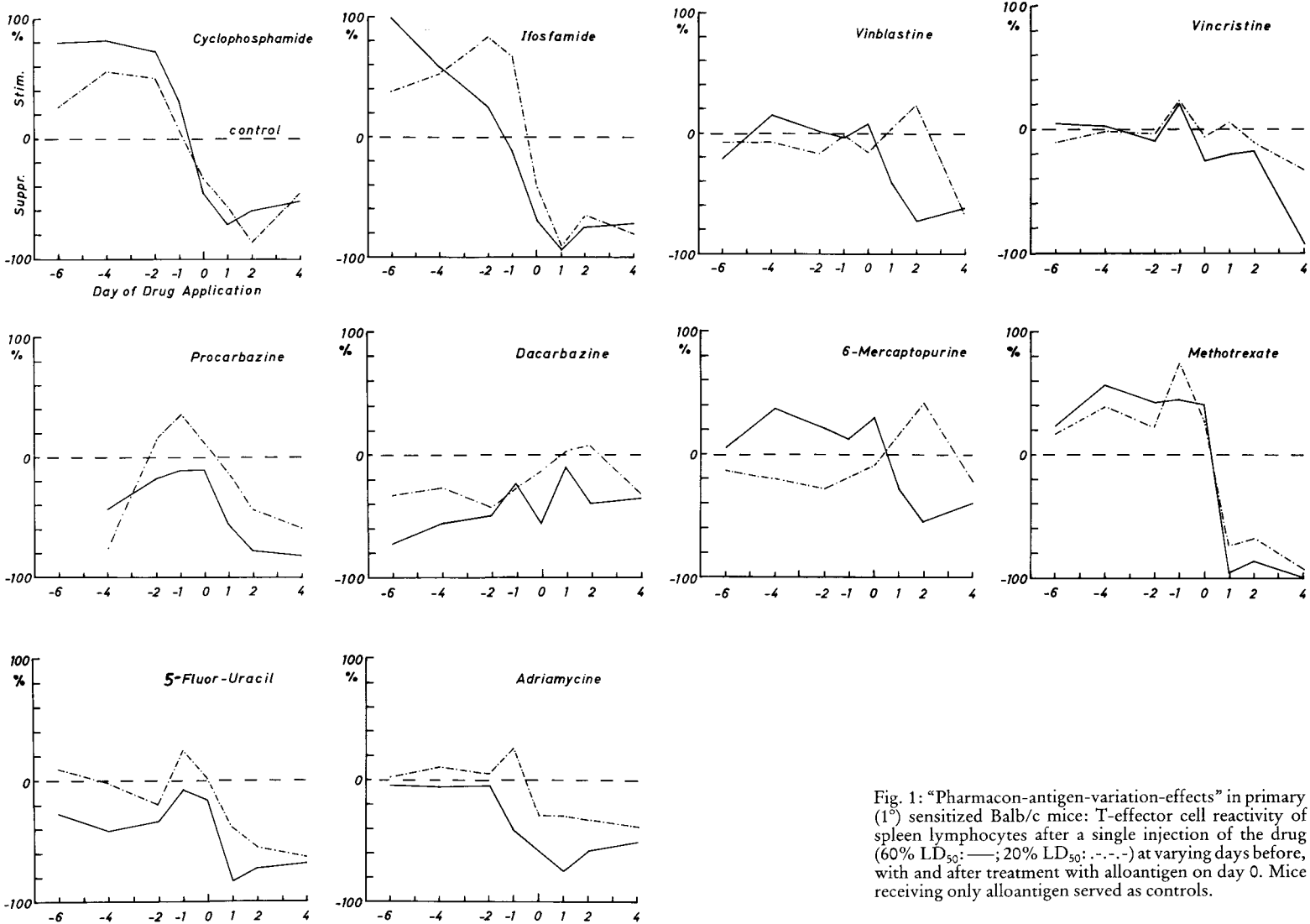

Fig. 1: "Pharmacon-antigen-variation-effects" in primary (1°) sensitized Balb/c mice: T-effector cell reactivity of spleen lymphocytes after a single injection of the drug (60% LD_{50}: ——; 20% LD_{50}: .-.-.-) at varying days before, with and after treatment with alloantigen on day 0. Mice receiving only alloantigen served as controls.

rocytotoxicity assay on day +5, the time of maximum reactivity of the untreated control group. In the following a short description will be given, analyzing the essential points of the reaction profiles of each of the investigated ten drugs.

Cyclophosphamide:
The reaction profile shows the form of an "inverted S shape" with strong stimulation when CY is applied before and strong suppression when applied with or after the antigen. The two dosages exert only a slight effect on the degree of stimulation.

Ifosfamide:
The reaction profile resembles that of the related substance CY, but with stronger suppression when IF is applied with and after the antigen. Compared to CY there are marked dose-dependent differences in the stimulation phase.

Vinblastine:
VLB has only a slight influence on T-effector cell reactivity when applied before or with the antigen. It causes a strong dose-dependent suppression when applied after the antigen.

Vincristine:
The reaction profile of VCR is very similar to that of the related alkaloid VLB: no significant immunomodulation in the phase before and strong dose-dependent suppression in the phase after the antigen.

Procarbazine:
PC produces a "bell-shaped" reaction profile with definite suppression when applied before and after but almost indifferent reactivity when applied shortly before or with the antigen. There is a dose-dependent difference in the level of the reactivity for most of the investigated days.

Dacarbazine:
DTIC exerts no characteristic modulating effect on T-effector cell reactivity. During the entire period of investigation, the curve rises gradually from strong suppression before to weak suppression after the antigen, without significant dependence on the dosage.

6-Mercaptopurine:
Application of 60% of the LD_{50} results in weak to medium stimulation before and with and moderate suppression after the antigen. The reaction profile for the 20% of the LD_{50} is almost a mirror image of this with weak suppression before and with the antigen, but partial stimulation after the antigen. For the first time not only quantitative, but also profound qualitative, dose-dependent differences occur in a reaction profile.

Methotrexate:
MTX has a reaction profile very similar to those of CY and IF ("inverted S shape") with significant stimulation before and with and very strong suppression after the antigen. There is no dose-dependence.

5-Fluor-Uracil:
FU produces a "bell-like" reaction profile with weak suppression before, indifferent reactivity with and strong suppression when applied after the antigen. The lower dosage causes a significant difference in the level of reactivity with an otherwise identical reaction profile.

Adriamycine:
AM influences the reactivity only slightly when applied before, but exerts medium to strong suppression when applied with or after the antigen. Pronounced dose-dependent differences are seen when it is applied shortly before and with the antigen.

2. *Secondary sensitization*

Figure 2 shows the PAVE for T-effector cell reactivity of 2° sensitized Balb/c mice. In addition to the treatment protocol described above the animals received two identical immunizations with C3H lymphocytes at a week's interval (on day −17 and −10) before re-immunization with the antigen on day 0. T-ef-

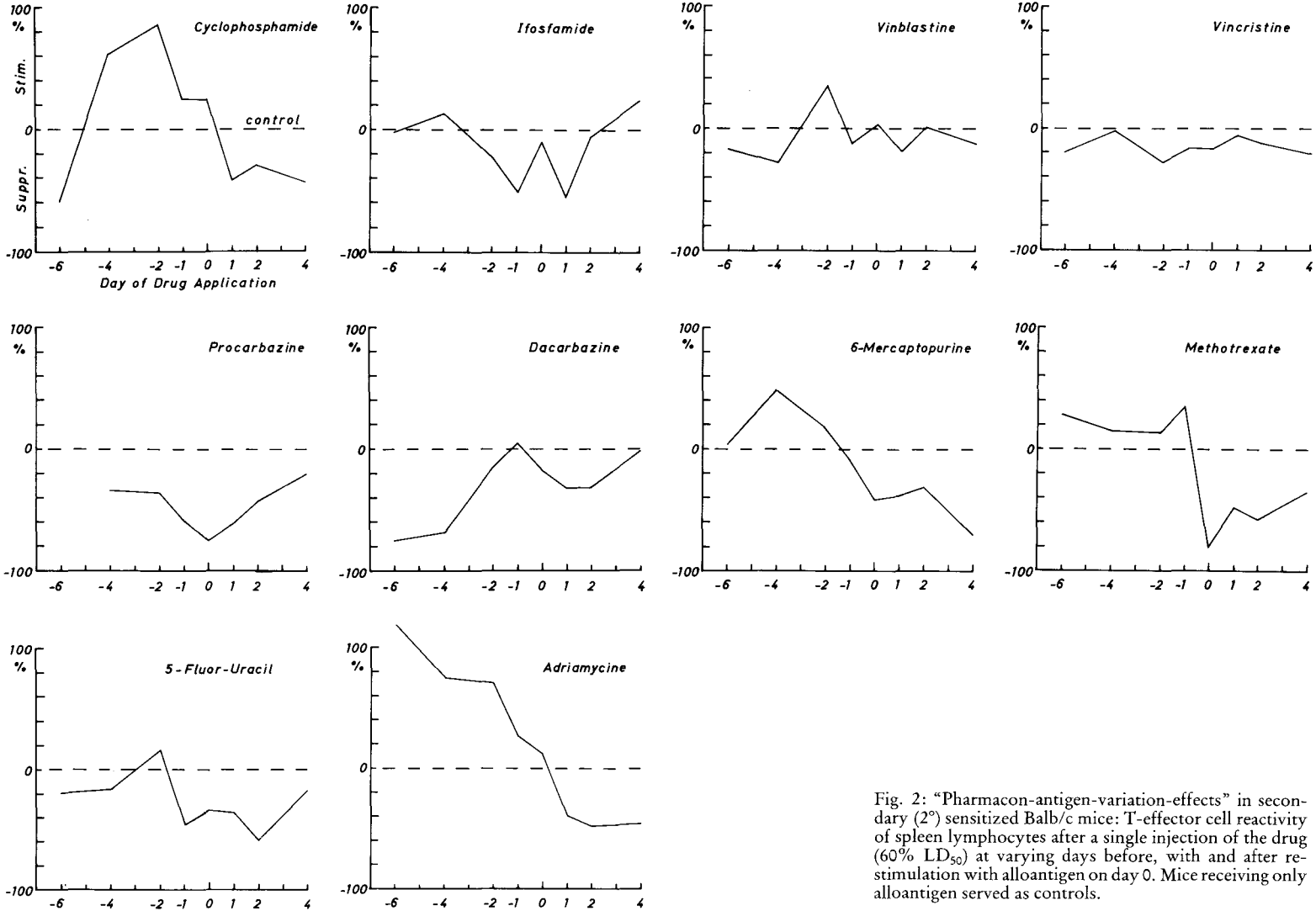

Fig. 2: "Pharmacon-antigen-variation-effects" in secondary (2°) sensitized Balb/c mice: T-effector cell reactivity of spleen lymphocytes after a single injection of the drug (60% LD_{50}) at varying days before, with and after restimulation with alloantigen on day 0. Mice receiving only alloantigen served as controls.

fector cell reactivity was tested on day +7, the time of maximum reactivity of the untreated control group.

Cyclophosphamide:
In comparison with the reaction profile for 1° sensitization, the stimulation before and with the antigen is more time-limited and the suppression after the antigen is less severe. In contrast to the reaction profile described above, there is a strong suppression on day −6 shortly after the last presensitization (the so-called "post 1° sensitization phenomenon").

Ifosfamide:
In comparison with the reaction profile for 1° sensitization one observes a totally different curve with "intrasuppressive" modulation. With the exception of day 0, the profile may be described as an "inverted bell-shape".

Vinblastine:
In contrast to the reaction profile for 1 ° sensitization, no significant modulation of the 2° immune reactivity occurs during the entire period of investigation.

Vincristine:
In correspondance with VLB we find indifferent reactivity, whether VCR is applied before, with or after the antigen. VCR appears to be ineffective in modulating 2° immune reactivity.

Procarbazine:
Compared with the reaction profile for 1° sensitization the "bell shape" is reversed, i.e., medium to weak suppression occurs before and after and strong suppression when PC is applied with the antigen.

Dacarbazine:
The reaction profile for 2° sensitization is similar to that of 1° sensitization with immune reactivity rising from strong suppression before to indifferent reactivity when DTIC is applied after the antigen. On days −6 and −4 there appears presumably a "post 1° sensitization phenomenon."

6-Mercaptopurine:
Similar to the reaction profile for 1° sensitization, we find an "inverted S shape" with weak to medium suppression before and medium to strong suppression after the antigen.

Methotrexate:
As for 1° sensitization, stimulation is observed before, suppression with and after the antigen. Both however, are less pronounced. The reaction profile resembles the "inverted S shape" of CY, IF and 6-MP.

5-Fluor-Uracil:
In comparison to the profile of 1° sensitization, FU 2° causes a stronger "intrasuppressive modulation". The reaction profile might also be described as an "inverted bell shape".

Adriamycine:
AM 2° shows a reaction profile with very strong stimulation before, indifferent reactivity with and medium suppression when applied after the antigen. The reaction profile resembles those of CY, IF, MTX and 6-MP in 1° sensitized mice; it is, however, remarkably different from that of AM in 1° sensitized animals.

3. *Classification of the reaction profiles according to reaction types*

On the basis of similarities between the reaction profiles of different drugs, an attempt was made to classify the substances with corresponding curves according to different reaction types. Regarding the reactivity *before*, *with* and *after* the antigen treatment on day 0, all reaction profiles can be classified according to one of the following 6 types (Table 1):

Type I – the "inverted S shape": It is characterized by strong stimulation before and strong suppression after the antigen, but mainly indifferent reactivity when the drug is applied together with the antigen. It occurs with CY (1°), IF (1°), 6-MP (1°, 2°), MTX (1°, 2°), and AM (2°).

Table 1 Classification of the reaction profiles of a variety of drugs (abbreviations see text) in 1° and 2° sensitized Balb/c mice into six reaction types depending on the modulation of immune reactivity before, with and after alloantigen treatment

Type 1 	before +++ with – after –––	CY 1°, MTX 1°, 2° IF 1°, AM 2° 6-MP 1°, 2°
Type 2 	before 0 with 0 after ––	VLB 1° VCR 1°, AM 1°
Type 3	before 0 with 0 after 0	VLB 2° VCR 2°
Type 4 	before –– with + after ––	PC 1°, FU 1° CY 2°
Type 5 	before – with –– after –	PC 2° IF 2°, FU 2°
Type 6 	before –– with – after 0	DTIC 1°, 2°

Type II – a "horizontal line with a downward bend": There is indifferent reactivity when the drug is applied before and with but increasing suppression when applied after the antigen. It occurs with VLB (1°) and VCR (1°) and less pronounced with AM (1°).

Type III – a "horizontal line": There is indifferent reactivity during the entire period of investigation. It occurs with VLB (2°) and VCR (2°).

Type IV – the "bell shape": It is characterized by suppression before and after and indifferent reactivity to weak stimulation when the drug is applied with the antigen. Examples are PC (1°), CY (2°) and FU (1°).

Type V – the "inverted bell shape": It is characterized by weak suppression before and after and strong suppression when the drug is applied with the antigen. It occurs with PC (2°) and less pronounced with IF (2°) and FU (2°).

Type VI – an "intrasuppressively rising line": It is characterized by strong suppression before, moderate suppression with and weak suppression when the drug is applied after the antigen. It occurs only with DTIC (1°, 2°).

Discussion

In contrast to considerable knowledge about the network of cell-mediated immunoregulation occurring during an immune response (*Germain* and *Benacerraf*, 1981; *Golub*, 1981) we still know very little about the way in which immunomodulating agents may influence these processes. Preliminary results have been reported for some substances, e.g., such as dexamethasone (*Larsson*, 1980), cyclophosphamide (*Turk* and *Parker*, 1982), adriamycine (*Ehrke* et al., 1982) and cyclosporin A (*Britton* and *Palacios*, 1982), but altogether our knowledge in the field of drug-manipulated immune reactivity is still very limited. This certainly contributes to the limitation of clinical success rates in the treatment of autoimmune diseases and graft rejection reactions. The necessity of studying both, pharmacological as well as immunobiological parameters in the application of new, but also of seemingly established substances in a more systematic and clearly comparative way led us to design the concept of a combined treatment of drug plus alloantigen to establish pharmacon-antigen-variation-effect (PAVE) patterns. From the two main variables, drug and antigen, the influence of the following parameters was studied in detail: (1) type of drug, (2) time of drug-application in relation to the antigen, (3) drug-dosage and (4) state of sensitization of the recipient. Although combined treatments have been described by several authors (*Berenbaum*, 1979; *Gaal* and

Nowotny, 1979; *Cottney* et al., 1980; *Goto* et al., 1981; *Turk* and *Parker*, 1982), systematic-comparative immunopharmacological studies for the T-cell system have so far not been reported.

The significance of the investigated immunobiological parameters for manipulation of immune reactivity according to the PAVE described above will be discussed in the following.

1. Type of drug

Although each drug shows its own distinct reaction profile expressing immunostimulation and/or immunosuppression certain characteristics of different immunomodulating drugs appear to correspond to each other. This led to their classification into one of the six reaction types described in Table 1. These reaction types do not correlate exactly with the biochemical modes of drug action (e.g. alkylating agent, alkaloid, antimetabolite, cytostatic antibiotic) although certain correlations are obvious. Thus, reaction type I, the "inverted S shape" is found primarily with alkylating drugs and antimetabolites, reaction type II and III primarily with the vinca alkaloids and reaction type VI only with dacarbazine. In spite of this, the a priori assumption of similar immunomodulating abilities of drugs belonging to the same biochemical group is not correct. Adequate characterization of drugs should thus include both, the biochemical as well as the immunobiological pattern of action. Especially, the hitherto often too single classification of drugs as immunosuppressive or immunostimulative appears to require reevaluation. (Furthermore: The recent tendency to use the terms immunostimulation and immunomodulation synonymously may create additional confusion.)

2. Time of drug application

This parameter appears to be essential for the characterization of an immunomodulating agent. Only by systematically varying this parameter is it possible to establish reaction profiles as shown in Figures 1 and 2. These may give hints for a distinct manipulation of immunoregulation and also for the underlying mode of action. Considering that the main effects of cytostatic drugs cannot simply be assumed to be nonspecific cytotoxicity, it should not be too surprising that very few drugs (DTIC 1°, 2°, FU 2°) show immunosuppression throughout the entire period of application, as would be expected from general impairment of proliferation. Obviously, so-called cytostatic drugs may well affect the immune response not only by differential cytotoxicity but also by a distinct interference with the immunoregulation. This is seen most strikingly with reaction type I in the form of strong immunostimulation.

3. Drug dosage

Compared with the other parameters investigated, the influence of the drug dosage on the reaction profile is surprisingly small. With most drugs only slight quantitative differences occur, whereas profound qualitative differences are rare (6-MP 1°, AM 1°). This result is remarkable because other authors have published data indicating that the dosage may play an important role in the manipulation of the immune response (*Orsini* et al., 1977; *Cottney* et al., 1980). Most authors however, express the dosage in terms of mg/kg body weight. In pharmacologically adequate terms it is not possible to compare results achieved on this basis. Therefore, our data, expressing in % of the LD_{50} and thus providing information on the actual toxicity of the drug in the animal, unfortunately allow for direct comparisons almost only between the various own findings.

4. State of sensitization

Beside the time of drug application, this parameter has a crucial influence on the resulting reaction profiles. Only for MTX, 6-MP and DTIC do the reaction profiles for 1° and 2° sensitized mice correspond to each other. In contrast, for CY, IF, VLB, VCR, PC, AM and FU the reaction profiles change completely. In the literature too little attention has so far been paid to the significance of the state of sensitization of the recipient. Experiments are nearly always carried out in 1° sensitized animals, where immune reactivity is built up for the first time by the antigen on day 0. In a presensitized organism however, the antigen boosters preestablished immune reactivity that will be altered by the application of the drug. Differences in the effects of cytostatic drugs in 1° and 2° sensitized organisms may thus provide information on the ability of a drug to interfere differently with different subsets of immunologically reactive cells.

Immunobiological mechanism of drug action

The modulation of the T-effector cell reactivity as presented here for the different investigated drugs directly leads to the question of the underlying mode of drug action. In view of the well-known network of T-regulator cells, analysis of this T-effector cell reactivity should include investigations of such regulating cells. Investigations of this kind are at present under study. First results indicate that some of the drugs not only activate or eliminate specific T-effector cells but also activate or eliminate specific resp. nonspecific T-suppressor cells depending on the application of the drug before or after the antigen. These preliminary findings are under further study; but it may already be stated that they will provide further insight into the remarkably differential effects of immunomodulating drugs.

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