Immunology, 1964, **7**, 511.

Tests for the Determination of the Effect of Antimitotic Products on Immune Reactions*

J. L. AMIEL, G. MATHÉ, M. MATSUKURA, A. M. MÉRY, G. DAGUET, R. TENENBAUM, S. GARATTINI, C. BRÉZIN AND V. PALMA

Cancerological and Radiopathological Research Centre of the Claude-Bernard Association, Hôpital Saint-Louis, Paris

(*Received* 15*th October* 1963)

Summary. The differences reported by various authors in regard to the inhibitory effect of the same antimitotic product on the production of antibodies, may be attributable in particular to the antigen used and to the technique of administration of the product. These differences have made it necessary to work out a test that is not subject to criticism in these respects.

After an investigation of the cytological reactions provoked in the mouse by various antigens, the authors have singled out three of these antigens: human albumin, the poliomyelitis virus and an allogeneic skin graft. The hyperbasophilic cells which are predominantly present in the lymphoid centres are, respectively, lymphocytes for the first, plasmocytes for the second and histiocytes for the third of these antigens.

In view of the different mechanisms of action that are possible with the different antimitotic products the authors used three methods of administration: entirely 'before' the antigenic stimulus; total dose administered entirely 'after' the antigenic stimulus; total dose administered partially 'before' and partially 'after' the antigenic stimulus.

INTRODUCTION

It may be expected that in the future, in the clinical utilization of antimitotic agents, their depressive effect on the immune defences will play a highly important part. These substances are already being used for experimental purposes (Calne, 1961; Weston, Maxwell, Lee, Finzel and Fisken, 1957; Zukoski, Lee and Hume, 1961) and in the clinical treatment of human patients (Kuss, Legrain, Mathé, Nedey and Camey, 1963) to facilitate allogeneic grafting; with the aid of these substances it has proved possible, in the animal, to suppress the secondary syndrome provoked by an allogeneic haematopoietic graft (Mathé, Amiel and Niemetz, 1962). These preparations may increase the possibilities of treatment of diseases due to auto-immunization (Dameshek and Schwartz, 1960; Lee, Meiselas, Zingalo and Richman, 1961; Milgrom and Witebsky, 1962; Schwartz and Dameshek, 1962).

The study of the action of these agents on the immune reactions is also of considerable theoretical interest, since its modalities and conditions allow an indirect analysis of the normal biological mechanisms which determine the production of antibodies; as a matter of fact, although a proliferation of cells with normal immunological function is a necessary stage in these reactions, the action of these chemical agents is not simply antimitotic.

* Investigation carried out with the aid of the European Coal and Steel Community, Contract No. 408, and the Centre d'Etudes Nucléaires (Fontenay aux Roses) Contract No. 60301 R.

Consequently, the systematic study of their depressive effects on the immune defences is nowadays an essential part of the 'screening' of products of which the possible antimitotic activity is to be determined.

We have found in the course of earlier investigations that, depending on the type of the antigenic stimulus, the type of the hyperbasophilic cells that proliferate varies (Mathé, Binet, Seman, Amiel and Daguet, 1963). It is essentially lymphocytes that are concerned in the case of a purified protein antigen (human albumin in the mouse), plasmocytes in the case of a virus antigen (the poliomyelitis virus) and histiocytes in the case of rejection of an allogeneic graft.

It is therefore necessary: (a) to test the product in question for each of these three types of immune reaction, since the cytological differences may reveal biochemical differences in the implicated processes, and consequently, different susceptibility to the various antimitotic agents; (b) to test these products by varying the time of their administration as related to the antigenic stimulus.

In this communication we shall report our attempts to develop a test which fulfils these requirements, and we shall report the results obtained with two antimitotic agents, the antimetabolite 6-mercaptopurine, and the alkylating agent cyclophosphamide.

MATERIALS AND METHODS

A. THE ANTIGENIC STIMULI AND THE IMMUNIZATION TESTS

(1) *Human Albumin*

The mice were given six intradermal injections, on alternate days, consisting of 0·1 ml. of a solution of human albumin, with a concentration of 10 mg./ml. Twelve days after the last injection of the product the immunization of these mice was studied with the aid of the test of clearance of human albumin labelled with radioactive iodine (Maguire and Mailbach, 1961).*

The dose injected intravenously into each mouse was 30 μc. The antigen clearance test was followed by daily determinations of the radioactivity in 0·1 ml. of circulation blood, taken by puncture of the sinus cavernosus (Lapeyraque, 1963). The radioactivity was expressed as a percentage of that measured 12 hours after the injection of the labelled albumin, and the mean daily percentages of each group of mice were plotted on a semi-logarithmic curve.

(2) *The Poliomyelitis Virus*

The mice were given six intradermal injections on alternate days of 0·1 ml. of a solution of 100×10^6 of the cytotoxic dose 50 per cent per ml. of virus of the strain 2 MEF_1, cultured on KB cells until their lysis, unfiltered and centrifuged for 15 minutes at 5000 ***g***. The immunization was evaluated 12 days after the last injection of the product by determination of the serum antibodies by the kinetic method of Lepine and Roger (1959). The antibody titres are given for 50 per cent protection. It has been demonstrated that in the mouse there exist no natural antibodies against this virus.

(3) *Allogeneic Skin Grafts*

The skin grafting was carried out by the method previously described by us (Tenenbaum,

* This albumin was supplied by CIBA, Brussels. The solution had the following characteristics: isotonic aqueous solution containing 15–25 mg. human albumin per ml.; the labelling consisted of one to two iodine atoms per protein molecule. The free iodine content did not exceed 2 per cent. The pH of the solution was between 8·5 and 9·5.

Méry, Amiel and Mathé, 1961); a flap of skin was taken from the back of the donor and placed on the tail of the host, and kept in place with a glass tube. The condition of the graft was determined every day or two by two observers, who did not know to what experimental group the animal belonged, until total necrosis of the graft had developed; the two criteria of which were a blackish colour and a total loss of suppleness. The results are expressed as the duration of survival of the grafts. Three genetic systems have been used successively: (a) grafts of skin from DBA/2 (H-2^d) donors on F_1 (CBA $\times$ C_{57}Br) (H-2^k $\times$ H-2^k) hosts; (b) grafts of DBA/2 skin on F_1 (CBA $\times$ C_{57}Br) which on the day of the skin grafting received an intra-peritoneal injection of 10^4 DBA/2 L 1210 leukaemic cells; (c) skin grafts from donors AkR (H-2^k) on F_1 (CBA $\times$ C_{57}Br) (H-2^k $\times$ H-2^k) hosts.

B. METHODS OF ADMINISTRATION OF THE ANTIMITOTIC PRODUCT

For each of these two products the LD_{50} with daily administration for 6 days was determined in the mouse. The animals of the experimental groups were given one-sixth of this daily dose for 6 days, then one-twelfth on alternate days for 2 weeks. The total duration of the treatment, therefore, was 20 days.

The antigenic stimulus, whether the first injection of heterospecific albumin or of the virus, or the application of the allogeneic graft, took place either on the 1st day of the treatment (treatment 'after' the stimulus), or on the 8th day (treatment 'before and after' the stimulus), or on the 20th day (treatment 'before' the stimulus).

(1) 6-*Mercaptopurine*

Since 'the LD_{50} for 6 days' daily administration was 144 mg./kg., the mice were given 24 mg./kg. every day for 6 days and 12 mg./kg. on alternate days for 14 days. This product was dissolved in a solution of 0·1 N NaOH, adjusted to a pH of 8–10 with diluted HCl.

(2) *Cyclophosphamide*

The 'LD_{50} for 6 days' successive administration was 255 mg./kg. and therefore the mice were given 42·5 mg./kg. per day for 6 days, and subsequently 21·25 mg./kg. on alternate days for 14 days. This product was dissolved in doubly distilled water.

C. THE EXPERIMENTAL GROUPS

(1) *Immunization against Human Albumin*

For each product to be tested, five groups of ten mice of Swiss stock, aged approx. 3 months, were used: group I, not immunized, received only the iodinated albumin; group II, immunized and not treated; group III, immunized and treated 'before'; group IV, immunized and treated 'before and after'; group V, immunized and treated 'after'.

(2) *Poliomyelitis Virus*

The mice were of Swiss stock, aged approx. 3 months; a group of nine mice, immunized and not treated, was kept as controls.

(a) 6-*Mercaptopurine.* Group I, immunized and treated 'before', eleven mice; group II, immunized and treated 'before and after', ten mice; group III, immunized and treated 'after', nine mice.

(b) *Cyclophosphamide.* Group I, treated 'before', nine mice; group II, treated 'before and after', ten mice; group III, treated 'after', ten mice.

(3) *Allogeneic Skin Grafting*

(a) Grafts of DBA/2 skin on F_1 (CBA $\times$ C_{57}Br) hosts. The donors and the hosts were of the same sex and were 3 months old.

6-*Mercaptopurine.* Group I, controls, not treated, twenty mice; group II, treated 'before', fifteen mice; group III, treated 'before and after', seventeen mice; group IV, treated 'after', fifteen mice.

Cyclophosphamide. Group I, controls not treated, fourteen mice; group II, treated 'before', fourteen mice; group III, treated 'before and after', twelve mice; group IV, treated 'after', twenty mice.

(b) Grafts of DBA/2 skin on F_1 (CBA $\times$ C_{57}Br) hosts, which on the day of the grafting were treated with 10^4 DBA/2 L 1210 leukaemic cells. One group was left untreated and kept as controls.

6-*Mercaptopurine.* Group treated 'before', ten mice; group treated 'after', ten mice.

Cyclophosphamide. Group treated 'before', six mice; group treated 'after', ten mice.

(c) Grafts of AkR skin on F_1 (CBA $\times$ C_{57}Br) hosts. A group of nine mice was left untreated and kept as controls.

6-*Mercaptopurine.* Group I, treated 'before', ten mice; group II, treated 'before and after', nine mice; group III, treated 'after', eight mice.

Cyclophosphamide. Group I, treated 'before', eight mice; group II, treated 'before and after', ten mice; group III, treated 'after', ten mice.

RESULTS

A. EFFECTS ON THE IMMUNIZATION AGAINST HUMAN ALBUMIN

(1) 6-*Mercaptopurine*

The results are shown in Fig. 1(a). There is no statistically significant difference in the antigen clearance between the immunized control group and the groups treated with 6-mercaptopurine.

(2) *Cyclophosphamide*

The results are shown in Fig. 1(b). There is not a statistically significant difference in the antigen clearance between the immunized control group and that treated with cyclophosphamide 'before' the antigenic stimulus. This clearence is, to a statistically highly significant degree, longer in the group treated with the product 'after' the antigenic stimulus. The antigen clearance in the group treated 'before and after' is intermediate between that of the group treated 'before' and that of the group treated 'after'; it differed from these two values to a statistically significant degree.

B. EFFECTS ON THE IMMUNIZATION AGAINST THE POLIOMYELITIS VIRUS

(1) 6-*Mercaptopurine*

The results are shown in Fig. 2(a). The statistical study of these results (Table 1) shows that the immunization is less good, to a statistically highly significant degree, in the group treated 'before' and to a significant degree, in the group treated 'before and after', than in the immunized untreated control group. On the other hand, there was no difference between the control group and the group treated 'after' the antigenic stimulus.

(2) *Cyclophosphamide*

The results are shown in Fig. 2(b). The statistical study of these results is summarized in Table 2. There are no statistically significant differences between the control group

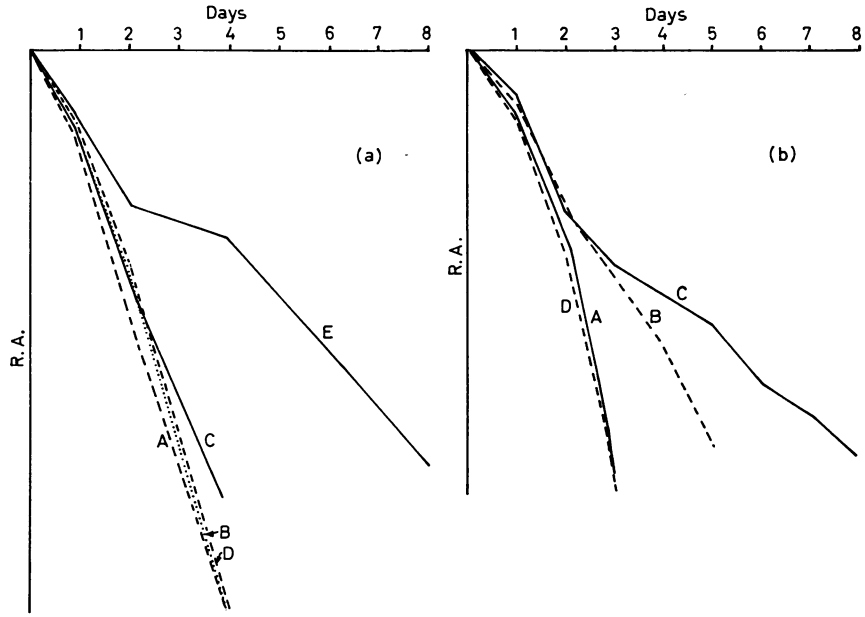

FIG. 1. Clearance of human albumin in mice immunized and treated with (a) 6-mercaptopurine and (b) cyclophosphamide. R.A. = radioactivity of the different groups of mice relative to radioactivity of day 1 (semi-logarithmic scale). A, treated 'before'; B, 'before and after'; C, 'after'; D, immunized controls; E, non-immunized controls. In (a) the three treated groups have as rapid clearance as the immunized untreated group. In (b) the group treated 'before' the antigenic stimulus has the same clearance as the immunized untreated control group. The group treated 'after' has a greatly decreased clearance. The group treated 'before and after' has an intermediary clearance.

TABLE 1

STATISTICAL COMPARISON OF THE EFFECT OF 6-MERCAPTOPURINE ON THE PRODUCTION OF ANTIBODIES AGAINST THE VIRUS OF POLIOMYELITIS DEPENDING ON THE TIME OF ADMINISTRATION OF THE PRODUCT IN REGARD TO THE TIME OF THE ANTIGENIC STIMULUS

	'Before'	*'Before and after'*	*'After'*
'Before'	—	$\chi^2 = 0{\cdot}38$ NS	$\chi^2 = 7{\cdot}58$ S for $P<0{\cdot}01$
'Before and during'	$\chi^2 = 0{\cdot}38$ NS	—	$\chi^2 = 4{\cdot}87$ S for $P<0{\cdot}05$
'After'	$\chi^2 = 7{\cdot}58$ S for $P<0{\cdot}01$	$\chi^2 = 4{\cdot}87$ S for $P<0{\cdot}05$	—

and the groups treated 'before' and treated 'before and after'; on the other hand, the immunization was to a statistically highly significant degree, less good in the group treated 'after' the antigenic stimulus.

C. EFFECTS ON THE REJECTION OF ALLOGENEIC SKIN GRAFTS

(1) *Grafts of DBA/2 (H-2^d) skin on F_1 ($CBA \times C_{57}Br$) (H-$2^k \times H$-2^k) hosts*

The results are shown in Fig. 3(a) for 6-mercaptopurine and in Fig. 3(b) for cyclophosphamide. There are no statistically significant differences between the control group and the various treated groups.

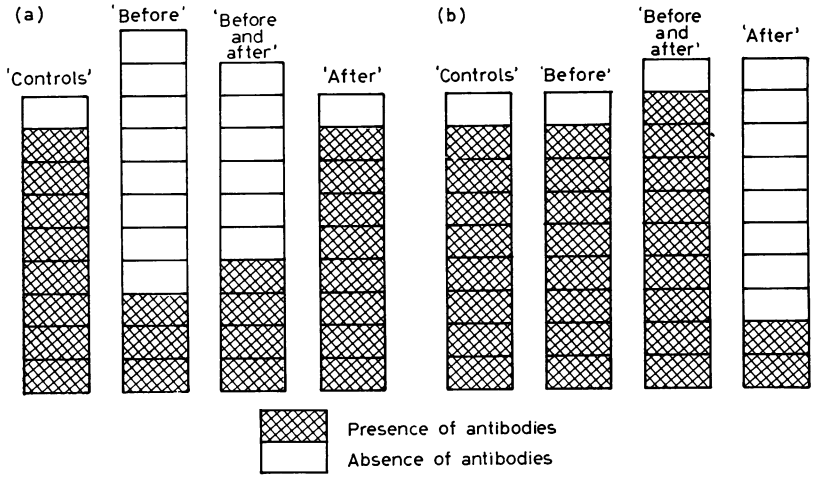

FIG. 2. Production of antibodies against the poliomyelitis virus in mice treated with (a) 6-mercaptopurine and (b) cyclophosphamide. In (a) the groups treated 'before' and 'before and after' the antigenic stimulus produce fewer antibodies than the control group. In (b) the group treated 'after' the antigenic stimulus produces fewer antibodies than the control group.

TABLE 2

STATISTICAL COMPARISON OF THE EFFECT OF CYCLOPHOSPHAMIDE ON THE PRODUCTION OF ANTIBODIES AGAINST THE VIRUS OF POLIOMYELITIS DEPENDING ON THE TIME OF ADMINISTRATION OF THE PRODUCT IN REGARD TO THE TIME OF THE ANTIGENIC STIMULUS

	'Before'	*'Before and after'*	*'After'*
'Before'	—	$\chi^2 = 0{\cdot}38$ for 1 d.f. NS	$\chi^2 = 6{\cdot}74$ for 1 d.f. S for $P<0{\cdot}01$
'Before and during'	$\chi^2 = 0{\cdot}38$ for 1 d.f. NS	—	$\chi^2 = 5{\cdot}06$ for 1 d.f. S for $P<0{\cdot}05$
'After'	$\chi^2 = 6{\cdot}74$ for 1 d.f. S for $P<0{\cdot}01$	$\chi^2 = 5{\cdot}06$ for 1 d.f. S for $P<0{\cdot}05$	—

(2) *Grafts of DBA/2 skin on F_1 ($CBA \times C_{57}Br$) hosts given 10^4 DBA/2 L 1210 leukaemic cells*

The results are summarized in Fig. 4. There are no statistically significant differences between the control group and the various treated groups.

(3) *Grafts of AkR (H-2^k) skin on F_1 ($CBA \times C_{57}Br$) (H-$2^k \times H$-2^k) hosts*

The results are shown in Fig. 5(a) for 6-mercaptopurine and in Fig. 5(b) for cyclophosphamide. The differences observed between the groups treated with 6-mercapto-

purine (Tables 3 and 4) and the untreated control group were not statistically significant; nevertheless, they seem to indicate a longer tolerance. The group treated with cyclophosphamide 'after' the antigenic stimulus shows, to a statistically significant degree, a longer tolerance than the control group or the groups treated 'before' and treated 'before and after' (Tables 5 and 6).

The combined results obtained with these three tests, using as antigenic stimuli, heterologous human albumin, poliomyelitis virus and an allogeneic skin graft, are summarized in Table 7.

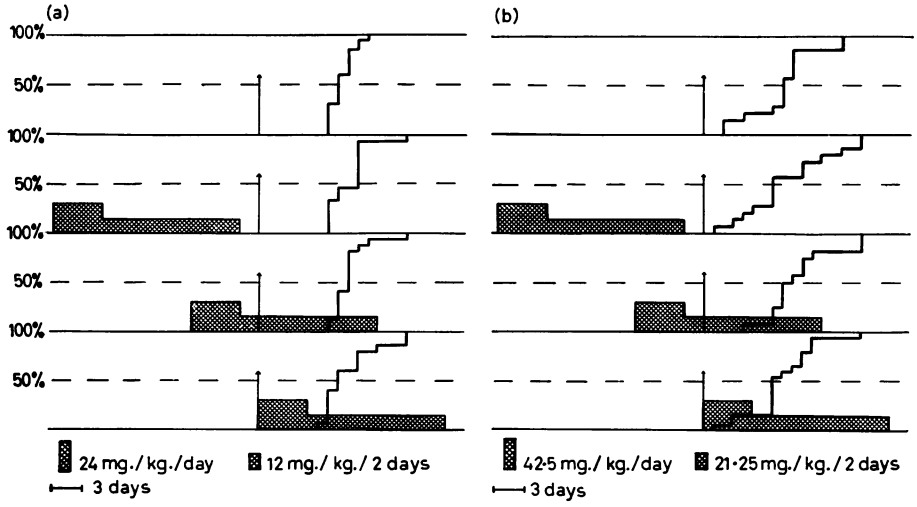

FIG. 3. Rejection of skin grafts (arrows) from DBA/2 (H-2^d) mice on F_1 (CBA × C_{57}Br) (H-2^k × H-2^k) hosts. The tolerance of these allogeneic grafts is not prolonged in the groups treated with (a) 6-mercaptopurine or (b) cyclophosphamide.

DISCUSSION

The purpose of this investigation was first of all to develop a method for the study of the inhibitory properties on the immune reactions of chemical treatments. Such a method has to be, on the one hand, simple and reproducible, and on the other hand, complete as well as sensitive.

The various tests that have been proposed for this purpose in the last few years have apparently not sufficiently fulfilled these different conditions.

In every test of the chemical inhibition of immune reactions, a number of variable elements can be summed up as follows: (a) the laboratory animal and its individual genetic characteristics; (b) the type of antigenic stimulus; (c) the duration and the intensity of the antigenic stimulation; (d) the methods of measurement of the immune reaction; (e) the type of the product used; (f) the dose administered and the duration of the treatment.

If a test is to fulfil the first-mentioned condition, i.e. that of being simple and reproducible, all these variable elements must be kept constant, with the exception only of the fifth element, the type of product used. With the various tests of the possibility of chemical inhibition of immune responses that have been described so far, this control is not possible; the conditions of the experiment vary not only from one author to another, but also they are not sufficiently standardized to be accurately reproducible. The animal material

shows great variation: the rabbit (Condie, Mennis and Miller, 1961; Green, 1958; Hoyer and Condie, 1962; Hoyer, Condie and Good, 1960; McQuarrie, Condie, Meeker, Roller and Varco, 1960; Nathan, Gonzale, Pescovitz, Jowles and Miller, 1960; Schwartz

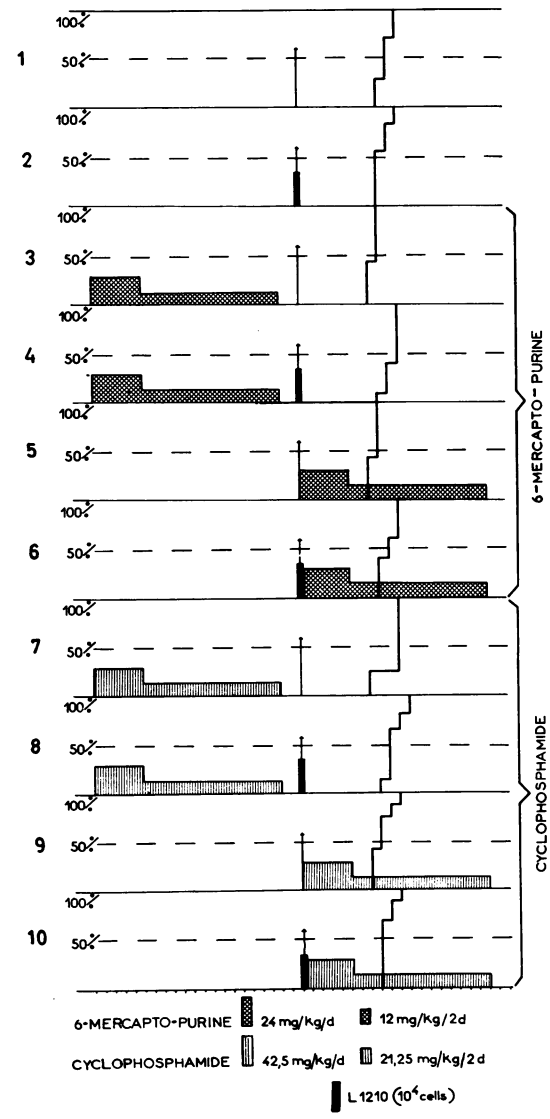

FIG. 4. Rejection of allogeneic skin grafts (arrows) of DBA/2 (H-2^d) mice on F_1 (CBA $\times$ C_{57}Br) (H-2^k $\times$ H-2^k) hosts. In the groups 2, 4, 6, 8 and 10 the animals were given, on the same days as the skin grafting, 10^4 DBA/2 L 1210 leukaemic cells. The tolerance of the skin grafts is not prolonged in the groups treated with 6-mercaptopurine or with cyclophosphamide.

and Andre, 1960; Schwartz, Eisner and Dameshek, 1959; Schwartz, Stack and Dameshek, 1958; Spurr, 1947), the mouse (Butler, 1961; Frisch and Davies, 1961; Humphreys, Chirigds, Milstead, Mantel and Goldin, 1961; Nathan, Bieber, Elion and Hitchings,

1961; Uphoff, 1958), the rat (Caskey, Moore, Tillotson and Hayman, 1951; McQuarrie *et al.*, 1960; Meeker, Condie, Weiner, Varco and Good, 1959; Weston *et al.*, 1957), the guinea-pig (Friedman, Buckler and Barons, 1961; Genghof and Battisto, 1961; Hoyer and Condie, 1962; Hoyer *et al.*, 1960; Maguire and Maibach, 1961b; Prichard and Hayes, 1961), the goat (Philips, Hopkins and Freeman, 1947), the chicken (Little, Oleson and

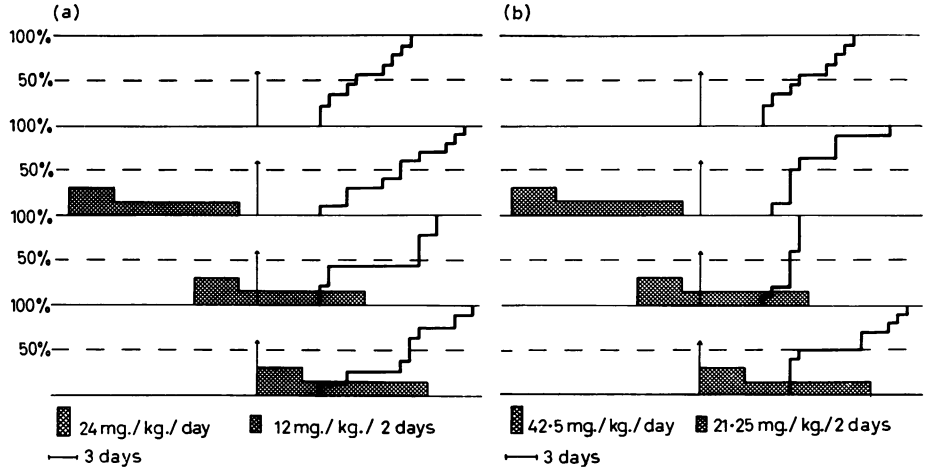

FIG. 5. Rejection of AkR (H-2^k) skin grafts (arrows) on F_1 (CBA $\times$ C_{57}Br) (H-2^k $\times$ H-2^k) hosts. The tolerance of the allogeneic skin grafts is prolonged in the three groups treated with 6-mercaptopurine (a), but the differences from the control group are not statistically significant. In the groups treated with cyclophosphamide (b) the tolerance of the group treated 'after' the antigenic stimulus is to a statistically significant degree longer than in the control group.

TABLE 3

SURVIVAL OF AkR SKIN GRAFTS IN F_1 (CBA $\times$ C_{57}Br) HOSTS; TREATMENT WITH 6-MERCAPTOPURINE

Controls	*No. of animals*	*Median survival (days)*	*Mean survival and confidence intervals for* P = 0·05		
Controls	9	11	9·2 days	11·7 days	14·2 days
Group treated 'before'	10	16	12·5 days	15·7 days	18·9 days
Group treated 'before and after'	9	18	9·9 days	13·8 days	17·7 days
Group treated 'after'	8	17	12·6 days	16·1 days	19·6 days

Roesch, 1950; Taliaferro and Taliaferro, 1948), the dog (Calne, 1961; Mannick, Lee and Egdahl, 1962; Pierce, Varco and Good, 1961; Zukoski *et al.*, 1961) and the monkey (Janssen, Marshall, Gerone, Cheville and Convey, 1961) have been used. Now, the same antigenic stimulus is not influenced in the same way by the same chemical treatment in every species, as has been demonstrated by Cole and Davis (1962), who have compared

TABLE 4

STATISTICAL COMPARISON OF THE EFFECT OF 6-MERCAPTOPURINE ON THE SURVIVAL OF AkR SKIN GRAFTS IN F_1 (CBA × C_{57}Br) (H-2^k × H-2^k) HOSTS, DEPENDING ON THE TIME OF ADMINISTRATION OF THE PRODUCT IN REGARD TO THE TIME OF THE ANTIGENIC STIMULUS

	Control group	*Group treated 'before'*	*Group treated 'before and after'*	*Group treated 'after'*
Control group	—	NS (t) = 1·82	NS	NS (t) = 1·95
Group treated 'before'	NS (t) = 1·82	—	NS	NS
Group treated 'before and after'	NS	NS	—	NS
Group treated 'after'	NS (t) = 1·95	NS	NS	—

TABLE 5

SURVIVAL OF AkR SKIN GRAFTS IN F_1 (CBA × C_{57}Br) HOSTS; TREATMENT WITH CYCLOPHOSPHAMIDE

Controls	*No. of animals*	*Median survival (days)*	*Mean survival and confidence intervals for* P = 0·05		
Controls	9	11	9·2 days	11·7 days	14·2 days
Group treated 'before'	8	10·5	9·6 days	12·5 days	15·4 days
Group treated 'before and after'	10	10		9·9 days	
Group treated 'after'	10	14·5	14·2 days	15·3 days	16·4 days

TABLE 6

STATISTICAL COMPARISON OF THE EFFECT OF CYCLOPHOSPHAMIDE ON THE SURVIVAL OF AkR GRAFTS IN F_1 (CBA × C_{57}Br) (H-2^k × H-2^k) HOSTS, DEPENDING ON THE TIME OF ADMINISTRATION OF THE PRODUCT IN REGARD TO THE TIME OF THE ANTIGENIC STIMULUS

	Control group	*Group treated 'before'*	*Group treated 'before and after'*	*Group treated 'after'*
Control group	—	NS	NS	S for $P<0·05$ (t) = 2·66
Group treated 'before'	NS	NS	NS	S
Group treated 'before and after'	NS	NS	—	S
Group treated 'after'	S for $P<0·05$ (t) = 2·66	S	S	—

the results in regard to the rejection of allogeneic skin graft, which were inhibited in the rabbit and not in the mouse after identical treatment with 6-mercaptopurine. Genghof and Batisto (1961) have further observed that the production of anti-bovine γ-globulin antibody is inhibited by 6-mercaptopurine in the rabbit, but not in the guinea-pig. With most of the animal species that have been used, it is not possible to carry out a genetic control sufficient to compare the results obtained by the various laboratories, or even the results obtained with different products in the same laboratory; actually, there are no pure lines in these species, a marked consanguinity which falls just short of complete homozygosity of the animals used (rats and rabbits) makes it even more difficult to interpret the results observed in the case of rejection of allogeneic grafts (McQuarrie *et al.*, 1960).

TABLE 7

INHIBITORY EFFECT OF 6-MERCAPTOPURINE AND OF CYCLOPHOSPHAMIDE ON THE IMMUNE REACTIONS, DEPENDING ON THE TYPE OF ANTIGENIC STIMULUS AND ON THE TIME OF ADMINISTRATION OF THE PRODUCT IN REGARD TO THE TIME OF THE ANTIGENIC STIMULUS

Product administered	*Administration of the antigens*			*Antigen*
	'Before'	*'Before and after'*	*'After'*	
6-Mercaptopurine	—	—	—	Human albumin
	+	+	—	Polio virus
	±	±	±	Allogeneic skin graft
Cyclophosphamide	—	±	+	Human albumin
	—	—	+	Polio virus
	—	—	+	Allogeneic skin graft

\+ Statistically significant.
± Positive but not statistically significant.

The type of antigenic stimulus also varies to a considerable degree from one experiment to another; bacteria or bacterial antigens (Laplante, Condie and Good, 1962; Little *et al.*, 1950; Spurr, 1947; Stender, Ringleb, Strauch and Winter, 1959; Sterzl, 1959, 1960a, b), virus (Cocchi, 1956; Potter and Haas, 1959; Sommers, Wilson and Hartman, 1951), heterologous proteins (albumins of various origin, γ-globulins, plant proteins administered with or without adjuvants) (Friedman *et al.*, 1961; Goh, Miller and Diamond, 1961; Laplante and Condie, 1960; Laplante *et al.*, 1962; Maguire and Maiback, 1961a; Philips *et al.*, 1947; Robinson and Christian, 1960; Schwartz and Andre, 1960; Schwartz *et al.*, 1958, 1959), heterologous or homologous red cells (Condie *et al.*, 1961; Frisch and Davies, 1961; Nathan *et al.*, 1961), allogeneic skin grafts (Cole and Davies, 1962; Levinson and Nechelas, 1956; McQuarrie *et al.*, 1960; Meeker *et al.*, 1959; Schwartz and Dameshek, 1962), renal transplantations (Calne, 1961; Nathan *et al.*, 1960; Zukoski *et al.*, 1961), allogeneic tumour grafts (Humphreys *et al.*, 1961; Preston, Jackson, Henegar and Schreck, 1960; Uphoff, 1958, 1961), allogeneic bone-marrow grafts (Weston *et al.*, 1957), secondary syndromes due to a reaction of the allogeneic haematopoietic graft to its host (Mathé *et al.*, 1962), appearance of an allergic encephalitis (Hoyer *et al.*, 1960), nephrotoxic nephritis (Caskey *et al.*, 1951), picryl chloride dermatitis (Genghof and Batisto, 1961), delayed sensitivity of the tuberculin type (Bukantz, Dammin, Johnson and Alexander,

1949; Hoyer and Condie, 1962). Most of these antigenic stimuli are not suitable for an accurate quantitative evaluation. As regards the other ones, the doses administered, the repetition of the doses, their spacing in time, differed from one experiment to another and particularly where the anti-bacterial immune reactions are concerned, it is often even difficult to be sure whether it is an immunitary reaction of first or of second set that is observed.

This variability of the antigenic stimuli, and accordingly of the immune reactions observed, sufficiently explains the different sensitivities of these reactions for the same product in the same animal. Even when the same antigenic stimulus is used, the intensity of this stimulus may determine the sensitivity of the immune reaction to the antimitotic product, as has been demonstrated by Nathan *et al.* (1961), who found that, paradoxically, the sensitivity is proportional to the intensity of the stimulus. On the other hand, it is clear that some of these antigenic stimuli that have been used are not suitable for a precise measurement of the immune reactions evoked, which are sometimes evaluated with the aid of highly indirect methods (André, Schwartz, Mitus and Dameshek, 1962).

Comparison of the efficacy of different products, with the same immune reaction, which is one of the purposes of the screenings, is possible only with standardization: (a) of the daily dose, determined on the basis of a biological effect that can be measured, and easily obtained with each of the products to be tested; (b) of the duration of the treatment. In the majority of the cases, this standardization has not been applied, and the doses have been selected arbitrarily, for instance, the doses of 6-mercaptopurine that have been administered to rabbits have varied from 1 mg./kg./day (Schwartz *et al.*, 1959), to 18 mg./kg./day (Hoyer and Condie, 1962). Furthermore, the doses administered are sometimes indicated very inaccurately, as 'the maximal tolerated dose', a dose with which, moreover, toxic effects have sometimes been observed, even to the point of the death of the animal in haematopoietic aplasia (Hoyer and Condie, 1962). The duration of the administration of the product has not been standardized either, since in some cases a single injection has been given (Berenbaum, 1960; Nathan *et al.*, 1961), whereas in other cases courses of treatment have been given that lasted as long as 21 days (Schwartz *et al.*, 1958), or even 30 days (Laplante *et al.*, 1962).

To sum up: the various studies in the field of chemical inhibition of the immune defences are difficult to compare with each other; in particular they are difficult to reproduce because of the lack of genetic control of the animal material used, because of the differences of the antigenic stimuli, and because of the arbitrary choice of the product in question and of the duration of the administration.

In our opinion, the test arrangement used by us in this work is free from such criticisms. The genetic control is ensured by the selection of the laboratory animal, viz. mice of Swiss stock, which have a sufficient genetic homogeneity for tests of immunization against a heterologous protein or a virus, and pure-bred mice for the tests with allogeneic skin grafts. The antigenic stimuli that we propose to use are quantitatively reproducible, and the methods used for the measurement of the immune reactions are sufficiently simple to be objective; finally the doses of the products to be tested are determined on the basis of a biological constant, the 50 per cent lethal dose for 6 days, which makes it possible to compare, for equivalent toxicity, the immune inhibitions brought about by the different chemical products.

The second requirement to be fulfilled by a test for screening of chemical substances for their inhibitory effect on immune reactions is that it must be sensitive, i.e. it must reveal

even a moderate effect of a product on the various types of immune reactions, and furthermore, it must be complete, i.e. it must also reveal a dissociated activity of a product on a particular type of immune reaction. It was in order to determine whether the proposed 'screening' fulfilled these two requirements, that we have studied, with this experimental system, two substances of which we already knew the activity on various types of immune reactions, viz. 6-mercaptopurine and cyclophosphamide.

The first test proposed, the clearance of a labelled human albumin, has given negative results with 6-mercaptopurine, which appears to be in contradiction to the experiments of Schwartz *et al.* (1959) and Schwartz and Andre (1960); however, their experiments have been carried out in the rabbit and not in the mouse; on the other hand, they have given positive results with cyclophosphamide. The second test, the production of antibodies that protect against the cytotoxic effect of the poliomyelitis virus, has given positive results with 6-mercaptopurine as well as with cyclophosphamide. The third test, the rejection of an allogeneic skin graft of a constant diameter, has not fulfilled this requirement of sensitivity when we have used donors and hosts that differed in the H-2 locus. In the mouse, Nathan *et al.* (1961), working with immunization against heterologous erythrocytes, have observed that the chemical inhibition of the immune reaction was proportional to the intensity of the antigenic stimulus; therefore, in a first stage, we have attempted to increase the sensitivity of this test by increasing the 'antigenic volume' represented by the allogeneic skin graft, by simultaneous injection of leukaemic L 1210 cells, obtained from the same allogeneic donors; the results were still negative. Our second attempt to increase the sensitivity of the test consisted in the selection of a genetic system in which the donor and the host did not differ in their major genes of histocompatibility, viz. those localized in the chromosomal region H-2; the results then were at the limit of statistical significance for 6-mercaptopurine, and positive for cyclophosphamide. Either of these tests, considered separately therefore, may be considered to be of sufficient sensitivity to be used in a 'screening' programme.

The last problem was to determine whether their variety is necessary and sufficient. We have considered it necessary to select them *a priori* because of earlier cytological studies (Mathé *et al.*, 1963), which demonstrated that in response to these three types of antigenic stimuli there occurred proliferation of different types of immunologically hyperbasophilic cells, lymphocytes, plasmocytes and histiocytes, so that different sensitivities to the same chemical agent might be the result.

The experiments that have been performed with 6-mercaptopurine and with cyclophosphamide confirmed that these three types of immune response are influenced in highly different ways by the same doses of the same product; 6-mercaptopurine, which is highly active in the mouse against the immune response to the poliomyelitis virus, is not active against the response to human albumin and is only slightly active against the rejection of an allogeneic skin graft. Accordingly, we are of the opinion that this variety of tests is necessary for a programme of 'screening' of chemical substances for their inhibitory effects on immune responses.

Finally, the chronological spacing of the administration of the product also plays an important part in regard to suppression of the immune reactions; the influence of this has been studied systematically, and this has only rarely been done in the earlier investigations, in which the majority of authors have attributed to all forms of chemical treatment a maximal activity in the 'inductive' phase of the antigenic stimulation (Hoyer *et al.*, 1960; Schwartz *et al.*, 1958; Shamaeva and Pankova, 1957; Stender *et al.*, 1959; Sterzl, 1960a, b;

Taliaferro *et al.*, 1948) and have often omitted to study the possible effect of the administration of the antimitotic substances prior to the antigenic stimulus. The investigation that has been most complete in this respect has probably been that by Schwartz *et al.* (1958) of the response to a heterologous protein in the rabbit treated with 6-mercaptopurine, but these authors have compared courses of treatment of different durations which were, moreover, 'asymmetrical' in regard to the day 0, the date of the antigenic stimulus (from day 0 to day +11, from day 0 to day +21, from day −7 to day +4, from day +7 to day +18, from day +18 to day +29).

Our investigations have shown that it is necessary to make a systematic study, for each product, of the effect on an immune response, of courses of treatment that are identical as regards dose and duration, but that are administered either before, or after the antigenic stimulus. Both 6-mercaptopurine and cyclophosphamide can suppress the immune responses of the mice to the poliomyelitis virus but 6-mercaptopurine is efficacious when it is administered 'before' the antigenic stimulus and inefficacious when it is administered 'after' the stimulus, the opposite is observed for cyclophosphamide.

The period during which a product must be administered to decrease the response to an antigenic stimulus, appears to depend on the product and not on the type of antigenic stimulus; cyclophosphamide decreases the immune response to three types of antigenic stimuli that have been tested, heterologous protein, poliomyelitis virus, and allogeneic skin graft. However, in these three cases it is efficacious only when it is administered 'after' the antigenic stimulus. The correlation between the moment when the product must be administered and the moment of the antigenic stimulus accordingly gives us an impression of the mechanism of action of the product on the immune response and, indirectly, of the mechanism of the immune response itself. The distinct difference between the moments when 6-mercaptopurine and cyclophosphamide must be administered in order to exert their action, may be considered in the light of recent findings concerning the control of the synthesis of specific proteins by cells of pluricellular organisms. Certain indirect arguments (Cohn, 1963; Kruh, Dreyfus and Shapira, 1962) suggest that the messenger RNA is stable in the cells of mammals. Once the messenger RNA necessary for the synthesis of specific antibodies has been formed, the cells that contain it can therefore continue to form the antibody proteins, even when the further synthesis of nucleic acid is inhibited by the presence of an antimetabolite of the molecules used in this synthesis, such as 6-mercaptopurine (Davidson, 1960). Therefore it is logical that 6-mercaptopurine exerts its maximal activity when it is administered 'before' the synthesis of this specific messenger RNA, i.e. before the antigenic stimulus. An alkylating agent such as cyclophosphamide, on the other hand, may affect the synthesis of the specific proteins through the messenger RNA, either by bringing about denaturation of the messenger RNA itself, or by causing denaturation of the various specific molecules, soluble or ribosomic RNA, or proteinic enzymes, that are necessary for this synthesis. Cyclophosphamide will therefore exert its maximal activity in the period of synthesis of the antibodies, i.e. when it is administered 'after' the antigenic stimulus, which is in accordance with our observations.

For all these reasons we are of the opinion that the above-named programme for the study of the inhibition of immune reactions brought about by chemical substances may be adopted for the 'screening' of the substances studied by the European Group of Anti-Cancer Chemotherapy: a single modification has been made: in the test of rejection of an allogeneic skin graft, we begin to study the effect of the product on the rejection of a graft from a donor which is not different from the host in its H-2 genes, and only in the

case of a positive result do we study the substance by the stricter test of rejection of an allogeneic skin graft containing H-2 antigens different from those of the host.

This systematic study has already given interesting results; in particular it has shown that a terephtanilide may act on an immune response regardless of whether it is administered 'before' or 'after' the antigenic stimulus, which observation suggests that the still unknown mechanism of the action of substances of this group differs from that of the antimetabolites and of the alkylating agents.

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