Biochemical Pharmacology, 1962, Vol. 11, pp. 29–44. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF CYTOTOXIC AGENTS ON THE PRODUCTION OF ANTIBODY TO T.A.B. VACCINE IN THE MOUSE

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(*Received* 31 *June* 1961; *accepted* 29 *July* 1961)

Abstract—Mice were given single injections of T.A.B. vaccine and nitrogen mustard, triethylene melamine, Myleran, 6-mercaptopurine, 6-thioguanine or amethopterin, or a single irradiation. It was found that irradiation and Myleran suppressed antibody production maximally if given about 2–4 days before the antigen, whereas the other agents did so when given about 2 days after the antigen. A single dose of agent given at the appropriate time depressed antibody production for some weeks. Once antibody production was well established it was relatively resistant to interference by these agents.

These findings suggest the hypothesis that differentation of the cells concerned in the immune response is accompanied by changes in their susceptibility to cytotoxic agents. During early stages of differentiation, before the antigenic stimulus has been received, they are sensitive to irradiation and Myleran. During the stage of differentiation that follows specific antigenic stimulation, they are sensitive to the other agents. When fully differentiated and engaged in producing antibody, they are relatively insensitive to agents of both classes.

INTRODUCTION

THE ability of certain toxic agents to interfere with the production of humoral antibody is well known. The most extensively studied of these is irradiation.[1, 2] Other agents of established effectiveness include mustard gas,[3] nitrogen mustards,[4] corticosteroids,[5] 8-azaguanine,[6] triethylene melamine[7] and 6-mercaptopurine.[8]

This action is not simply due to general toxicity; many drugs have no demonstrable effect on antibody production even in lethal doses, and effective agents may markedly inhibit antibody synthesis in doses not in the lethal range.[9, 10]

The specificity of the irradiation effect is also suggested by its close dependence on the relation between the times of irradiation and of antigen administration and this dependence has provided a useful means of studying the initiation of the immune response.[1, 2, 11, 15] It therefore seemed likely that an extension of these studies to other inhibitory agents would provide further insight into the mechanism of the antibody response and into the modes of action of these agents.

MATERIALS AND METHODS

Animals

Male albino mice, of a strain closely inbred from Strong A2 ancestors, were fed on M.R.C. diet no. 41. They weighed 15–22 g at the start of each experiment.

Irradiation

Whole-body irradiation was effected by Co^{60} γ-rays, 450, 500 or 600 r at a rate of 10 r/min. The dose-rate was calibrated by the Fricke ferrous sulphate dosimeter method for γ-ray doses in the range of 4–40 kr.

Chemicals

Sources. Nitrogen mustard (methyl bis (β-chloroethyl) amine) was obtained from Boots Pure Drug Co. Ltd., triethylene melamine from Imperial Chemical Industries Ltd., Myleran (1:4-dimethane sulphonoxybutane) batch 74985 from Professor F. Bergel of the Chester Beatty Research Institute and from Wellcome Research Laboratories, 6-mercaptopurine from Wellcome Research Laboratories, 6-thioguanine from L. Light and Co. Ltd. and amethopterin from Lederle Laboratories Ltd.

Injections. Water-soluble materials were dissolved in 0·85 per cent saline. Insoluble materials were well ground in a glass homogenizer in saline together with 0·5 per cent high-viscosity carboxymethyl cellulose (Hercules Powder Co.). Myleran was lightly ground in arachis oil. All agents were used within 1 hr of preparation and the alkylating agents in saline within 5 min. Injections were made subcutaneously into the lower back. The large volume of 1 ml/20 g mouse was used so as to reduce inaccuracies in dose when suspensions were injected.

Doses. These were determined in preliminary toxicity trials and were the maximum consistent with the survival of three or more mice out of each group of five. However, mortality varied greatly and unpredictably in different experiments. Particular difficulty was experienced with Myleran, probably owing to the different particle sizes of different batches. Accordingly, all the results given here for this drug refer to a particular batch of large particle size.

The doses were: nitrogen mustard, 2·4 mg/kg, triethylene melamine, 2 mg/kg, Myleran, 125 or 150 mg/kg, 6-mercaptopurine, 150 mg/kg, 6-thioguanine, 37·5 mg/kg and amethopterin, 17·5 mg/kg.

Antigen.

This was 0·2 ml of T.A.B. vaccine (Typhoid-paratyphoid A and B Vaccine, B.P.), given intraperitoneally.

Schedule of treatment

Each group consisted of five or, in irradiation experiments, seven mice. Animals were given a single injection of antigen and a single injection of toxic agent or a single irradiation. The day of antigen injection is referred to as day 0. Irradiations or injections of drugs were given to different groups each day between day − 5 and day + 8. Irradiations were also carried out at − 2 and + 2 hr and − 25 and + 25 min, measured from the mid-point of irradiation. Injections on day 0 were given 5 min before the vaccine. In most experiments sera were taken for titration on day + 9.

Sera

Mice were killed by a scissors cut below the base of the skull. Blood from each animal was allowed to clot separately, and equal volumes of serum from each mouse in a group were pooled. Groups in which less than three mice survived the treatment were discarded. Sera were kept at − 20 °C and titrated within 2 weeks of collection.

Titration

A Widal titration was performed with *S. typhi* antigen. Serum-antigen mixtures were kept at 52 °C for 1 hr and stood at room temperature for 30 min before reading.

Titres are expressed in this paper as reciprocals of the end-point dilution, and as powers of the base 2, i.e. an end-point dilution of 1 in 64 is here given as 2^6.

Statistical methods

Strictly speaking, the proper way to have interpreted the results would have been to compare, by the χ^2 test, the frequency with which the various titres were observed in the experiments with frequencies observed of the corresponding titres in the controls. However, in the experimental results most frequencies were less than 5, in which circumstances the χ^2 test is not applicable. For this reason, frequencies observed for all titres equal to or less than 2^4 were pooled, and similarly frequencies for all titres equal to or greater than 2^5 were pooled, in both experimental and control groups (consideration of Fig. 2 suggested this dividing line). Even then, frequencies observed were often less than 5. The significance of the resulting 2×2 table was therefore calculated directly from the hypergeometric distribution. This method to some extent underestimates the differences between experimental groups and controls, as it takes no account of the extent to which titres are below 2^4 or above 2^5.

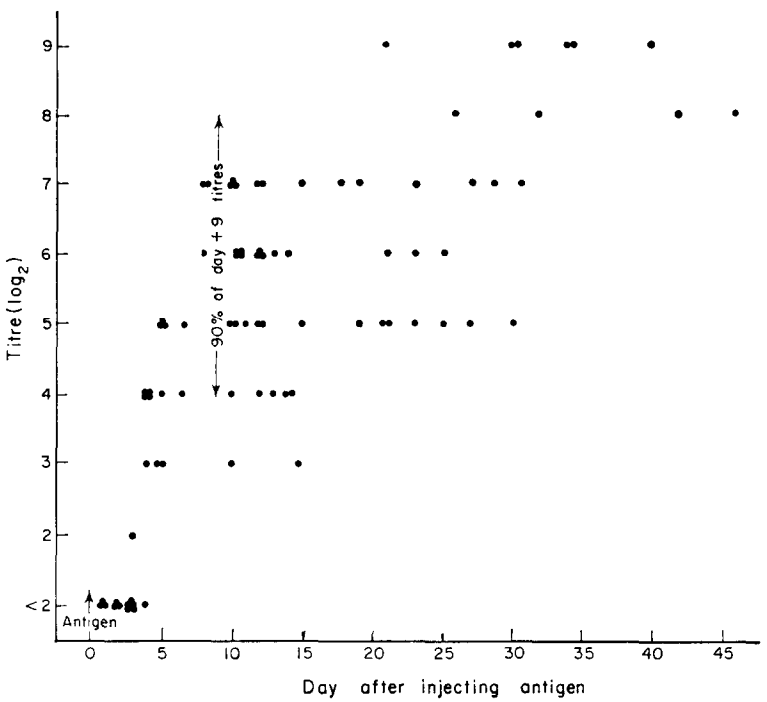

FIG. 1. Response of normal mice to T.A.B. vaccine, 0·2 ml i.p. Each point represents the pooled sera of five mice.

RESULTS

Response of normal mice to T.A.B. vaccine

Agglutinins appeared in the serum 4 days after injection and rose to a titre of about 2^6 by the eighth day. This titre was maintained for 2–3 weeks and then rose further (Fig. 1). Serum from fifty groups of mice were titrated 9 days after injection. The mean titre was $2^{5 \cdot 42}$ and 90 per cent of the titres lay between 2^4 and 2^8 (Fig. 2).

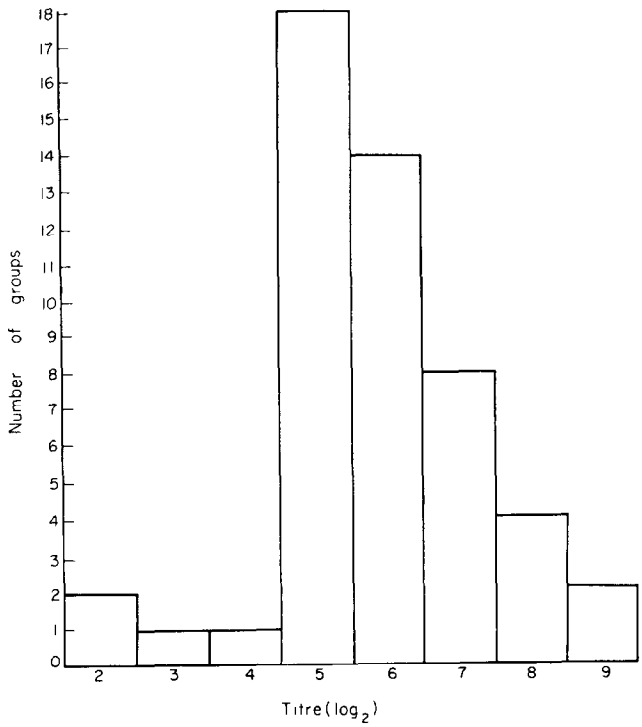

FIG. 2. Distribution of day + 9 titres in fifty groups of five mice after T.A.B. vaccine, 0·2 ml i.p.

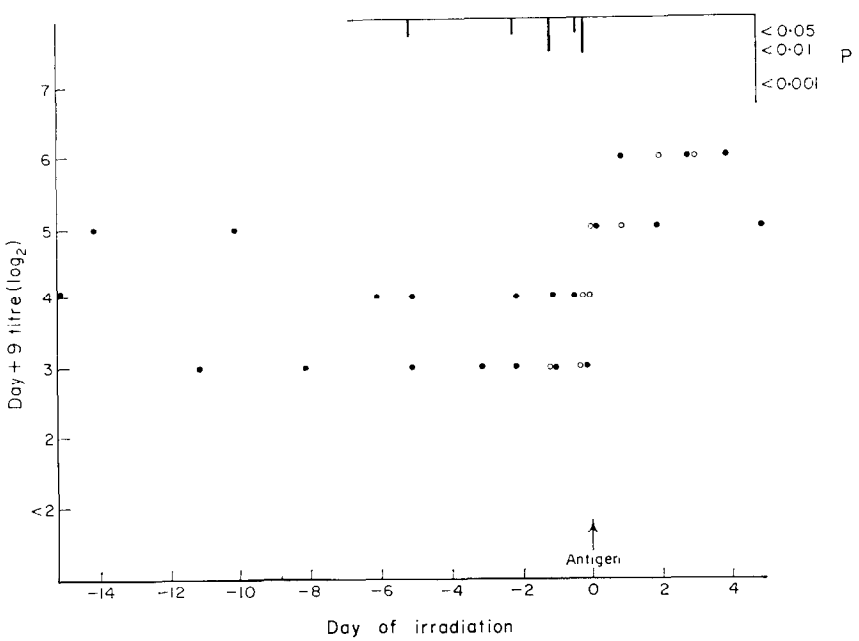

FIG. 3. Effect on day + 9 titre of irradiation with 450 r (○) or 500 r (●) before or after injection of antigen. Significance of the degree of depression is shown at the top of this and subsequent figures.

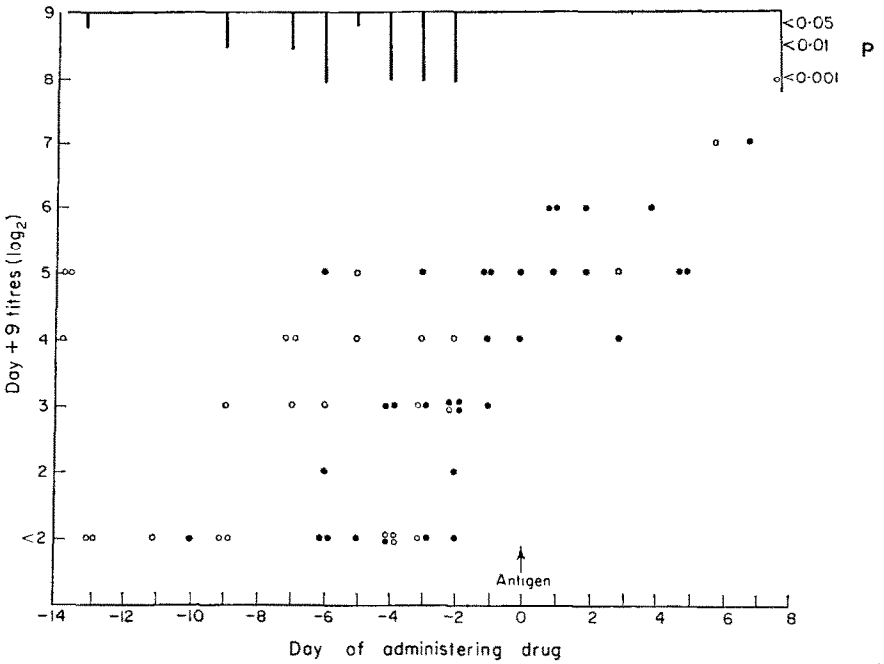

FIG. 4. Effect on day + 9 titre of Myleran, 125 mg/kg (○) or 150 mg/kg (●) s.c. before or after injection of antigen.

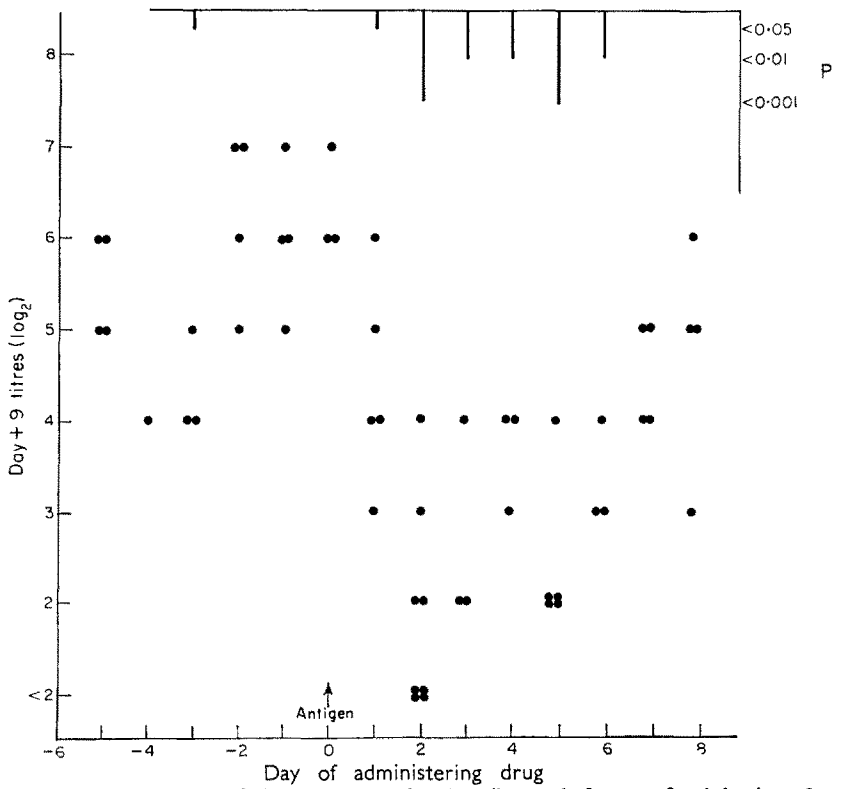

FIG. 5. Effect on day + 9 titre of nitrogen mustard, 2·4 mg/kg s.c. before or after injection of antigen.

Effect of irradiation

Irradiation with 450 or 500 r at + 2 hr or later did not affect the antibody level on day + 9. Statistically significant depression of titres was caused by irradiation given at times between day − 5 and + 25 min. Titres on day + 9 approached normal levels if the irradiation had been carried out 10 days or more before the injection of antigen (Fig. 3).

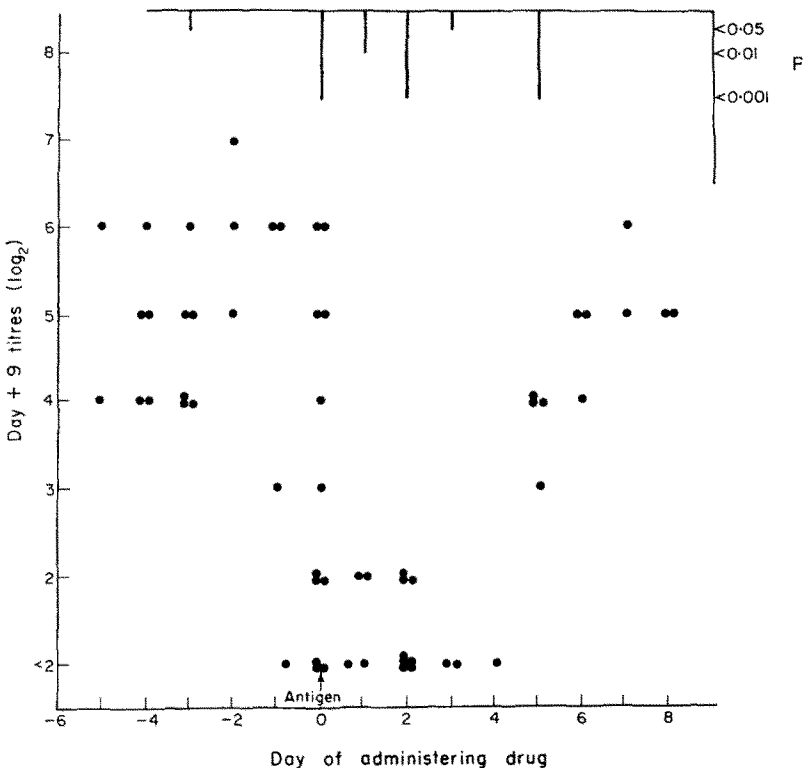

FIG. 6. Effect on day + 9 titre of triethylene melamine, 2 mg/kg s.c. before or after injection of antigen.

Effect of Myleran

Although there was a wide scatter of results, significant depression in the day + 9 titre resulted when the drug was given on days − 9 to − 2. Administration of Myleran on day − 1 or later did not reduce the titre (Fig. 4). The effect of Myleran was thus broadly similar to that of irradiation.

Effect of other agents

Nitrogen mustard, triethylene melamine, 6-mercaptopurine, 6-thioguanine or amethopterin each produced effects the reverse of those produced by irradiation and Myleran. Apart from an occasional group, little or no reduction in the day + 9 titre was observed in mice injected before administration of the antigen. Usually inhibition

resulted if the drug was given between day 0 and day + 6 and was most marked when the drug was injected on day + 2. Inhibition was progressively less effective as the time of administration of the drug was postponed from day + 2, and animals injected with these agents a day or so before bleeding showed normal or nearly normal antibody levels (Figs. 5, 6, 7, 8, 9). The results with these drugs were thus strikingly similar, in spite of the many differences between their modes of action, persistences and metabolic fates.

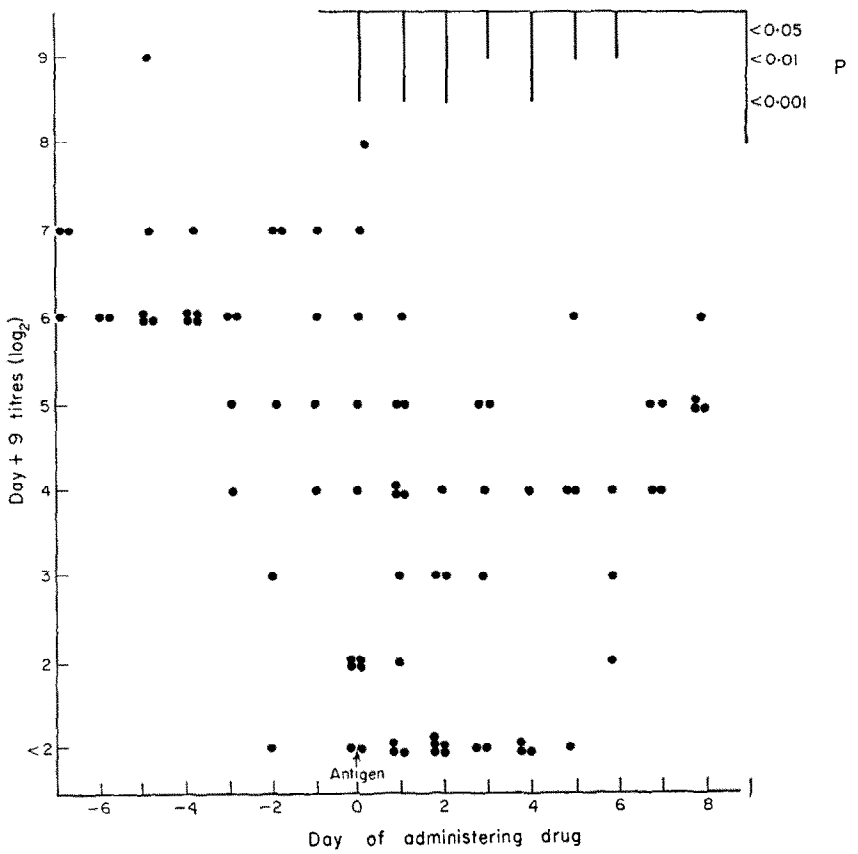

FIG. 7. Effect on day + 9 titre of 6-mercaptopurine, 150 mg/kg s.c. before or after injection of antigen.

Duration of inhibition

Animals were given nitrogen mustard, triethylene melamine, 6-mercaptopurine, 6-thioguanine or amethopterin on day + 2, or irradiated with 600 r on day − 2, and injected with vaccine on day 0 and groups were bled at intervals up to day + 50. The results are shown in Fig. 10. Most agents, when given at the appropriate time, depressed antibody levels for a month, after which time they began to rise slowly. Normal levels were not reached by day + 50 (compare Fig. 1). With triethylene melamine, titres tended to rise after 2–3 weeks, but again, apart from a single group on day + 25, normal levels were not attained during the period of observation.

Effect on established antibody levels

Mice were injected with various agents or irradiated 10 days after injection of antigen, sera being collected on subsequent days up to day + 17. Nitrogen mustard, triethylene melamine or thioguanine caused slight and transient falls in antibody levels, which recovered by day + 17. This effect was not observed with irradiation, Myleran, 6-mercaptopurine or amethopterin (Fig. 11).

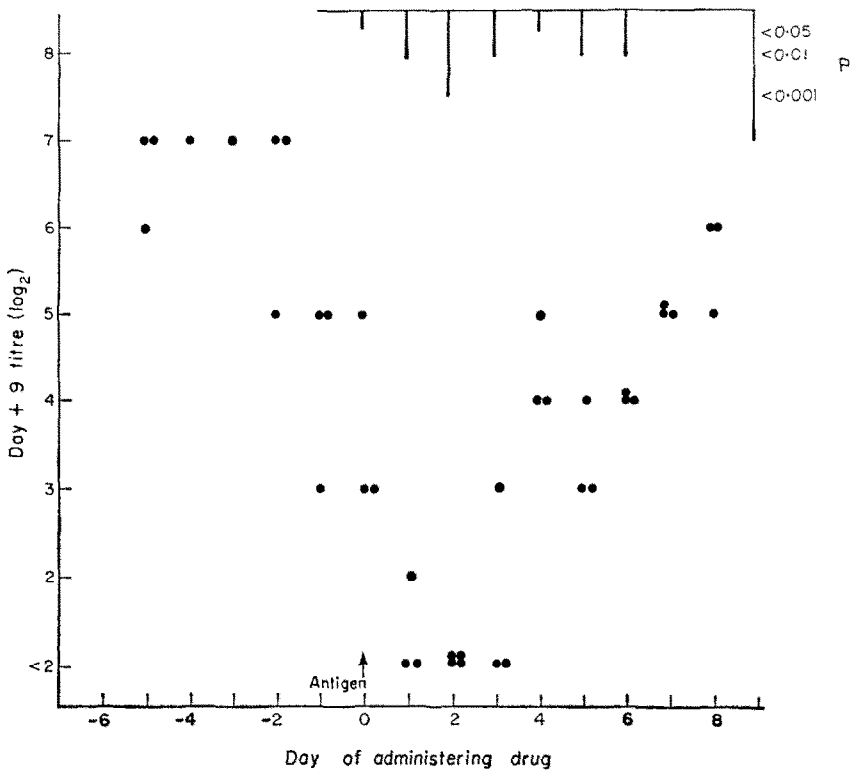

FIG. 8. Effect on day + 9 titre of 6-thioguanine, 37·5 mg/kg s.c. before or after injection of antigen.

DISCUSSION

Evidence of the complexity of the initial stages of the immune response is provided by comparing the effects of the various agents used in this work. Irradiation and Myleran, which will be referred to as class 1 agents, act if given before the antigen; the mustards and antimetabolites, which will be called class 2 agents, act if given after the antigen. It is difficult to account for these facts satisfactorily by a hypothesis specifying both the effective biochemical or morphological lesion produced by each agent and the stage of the immune response with which it interferes.

It is evident that the immune response is most sensitive to class 2 agents between 1 and 5 days after giving the antigen. The evidence is not so clear for class 1 agents. Some toxic materials may cause delayed damage because of their persistence or slow absorption. It might be claimed that agents depressing antibody production only if

given before the antigen are of this nature. It would be implied that in this event both classes of agent act on the same stage of the induction period of the immune response, class 2 agents acting with much the greater speed. However, this can hardly be said of irradiation, whose effects on such key processes as DNA synthesis and mitosis begin within hours. Such a claim might be made with more force for Myleran, although its rate of catabolism is much faster than that of amethopterin, a class 2 agent.[12–14] As will be shown below, there are other reasons for thinking that the mode of action of Myleran is similar to that of irradiation.

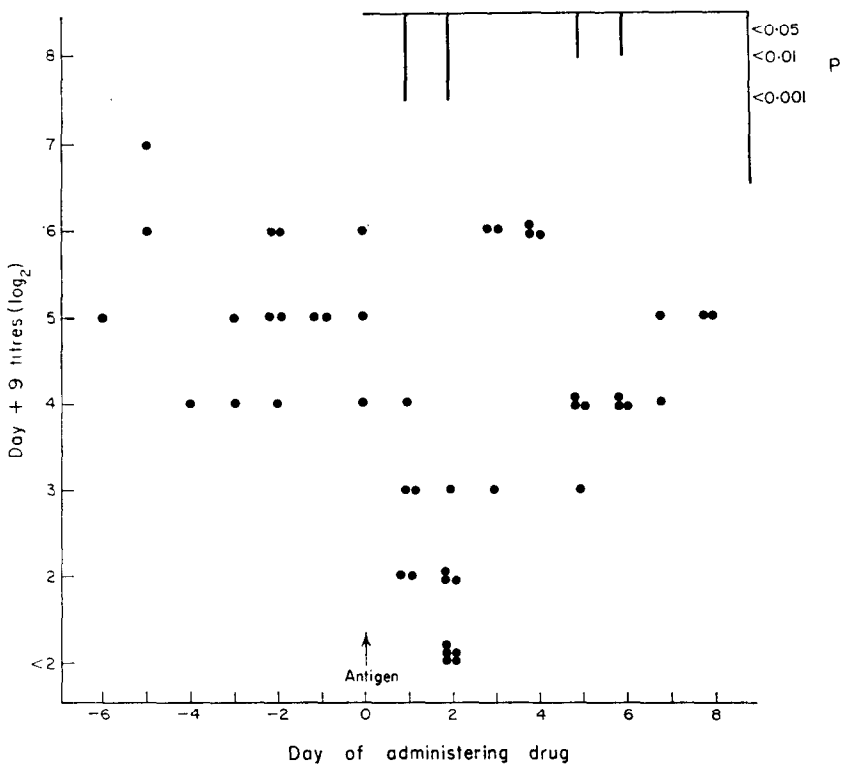

FIG. 9. Effect on day + 9 titre of amethopterin, 17·5 mg/kg s.c. before or after injection of antigen.

It may be that class 1 agents must be given before the antigen because they act on an earlier stage of the immune response than class 2 agents. Graphs relating the time of irradiation to levels of antibody production show an apparently abrupt ending of radiosensitivity of the immune response during the day before the antigen injection.[1, 2, 11] However, the affected cells possibly remain radiosensitive until some hours after injection of antigen for the known effects of irradiation take at least a few hours to reach their peak. Because of this and perhaps also because it may seem illogical to say that the immune response is radiosensitive before the antigen has been injected, that is, before the immune response has begun, it has been suggested that the radiosensitive stage begins shortly after the antigen injection and ends some hours later.[1, 2] However, this hypothesis does not readily account for the fact that irradiation can

impair antibody production even when given some weeks before the antigen[11, 15] and the alternative hypothesis outlined below is put forward.

Immunologically competent cells belong to a cell-line whose developmental history does not begin only with exposure to antigen. It is possible, as Talmage suggested,[16] that they become responsive to antigen only during a particular stage in their development, so that only a fraction of the cells of this line will respond at any one time to any given primary antigenic stimulus. An agent damaging the cell-line before this stage might therefore inhibit the response to a subsequent antigen injection by delaying or perverting the further development of the affected cells, by destroying them, by impairing their capacity to divide or by some other means. A parallel is suggested by

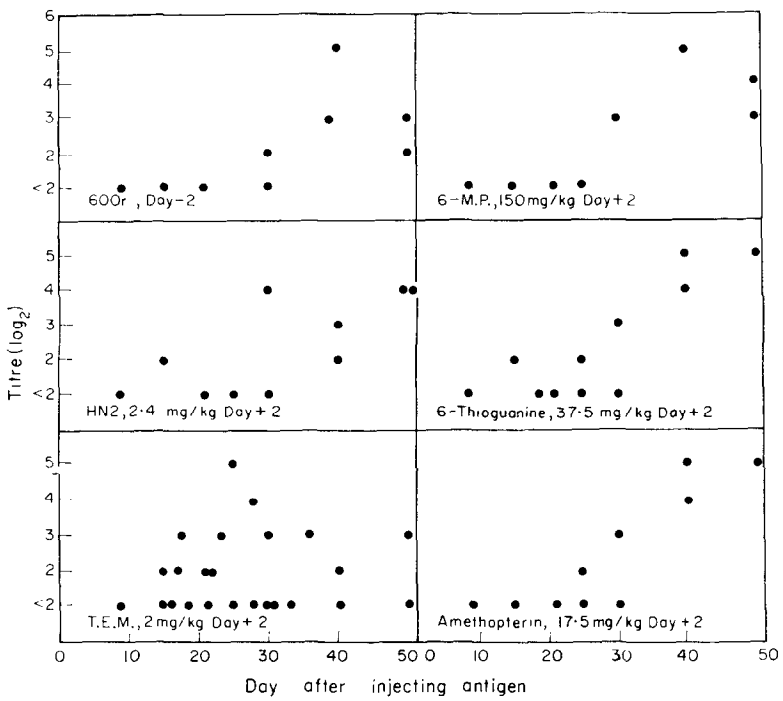

FIG. 10. Effect of irradiation on day − 2 and various chemicals on day − 2 on antibody levels followed up to day + 50.

the work of Albert and Bucher[17] on the proliferation of liver cells induced by partial hepatectomy. They found that cell-multiplication was inhibited by irradiation given as much as 4 weeks before hepatectomy. It is unlikely that such long-term effects were due to a special toxicity of persisting irradiation products for tissues stimulated to proliferate. It is more probable that the cells were damaged shortly after irradiation and long before they received the stimulus to divide. In the same way it is not likely that the stage of the immune process occurring immediately after exposure to antigen is so sensitive to persisting products of irradiation or Myleran that it can be inhibited by these agents even if they are given a week or more beforehand. It is more likely that damage is caused by these agents soon after they are given and is only made apparent by subsequent failure to respond to antigenic stimulation.

According to this hypothesis, therefore, the stage during which an immunologically competent cell is sensitive to class 1 agents begins some weeks before the time when it is maximally responsive to antigen and ends at about the time of exposure to antigen. The length of this stage is measured by the time taken for immunological responsiveness to return after treatment with these agents; it is a function either of the rate at which the cells concerned are replaced by division or differentiation of precursors or of the rate at which damage by irradiation or Myleran is repaired in these cells.

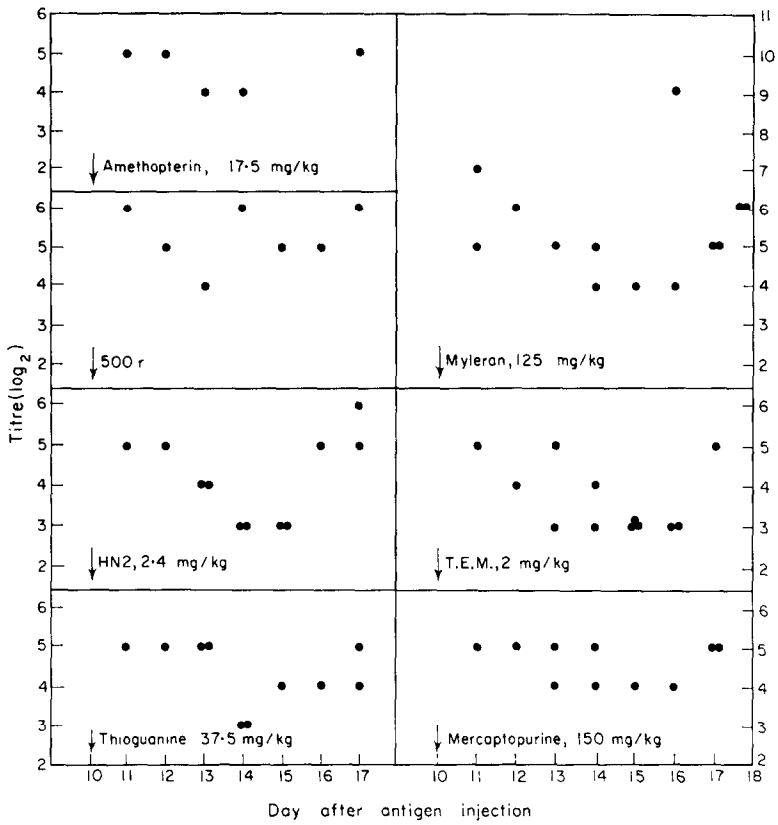

FIG. 11. Effect on subsequent antibody levels of agents given on day + 10.

There is evidence, from other experimental systems, that class 1 and class 2 agents affect different stages in the development of a cell-line. This is shown most clearly by the work of Jackson and his colleagues on spermatogenesis in the rat.[18, 19] Some of their findings are indicated by Fig. 12. It appears from this that developing sperm-cells, in their early stages, are most sensitive to irradiation and Myleran and, in their late stages, to triethylene melamine. Allowing for the difference in time-scale, the general resemblance between Fig. 12 and Figs. 3, 4 and 6 is striking. Moreover, it will be noted that sensitivity to particular agents is here a function of cell-differentiation rather than of cell-division; maximal sensitivity to Myleran occurs before cell-division begins and

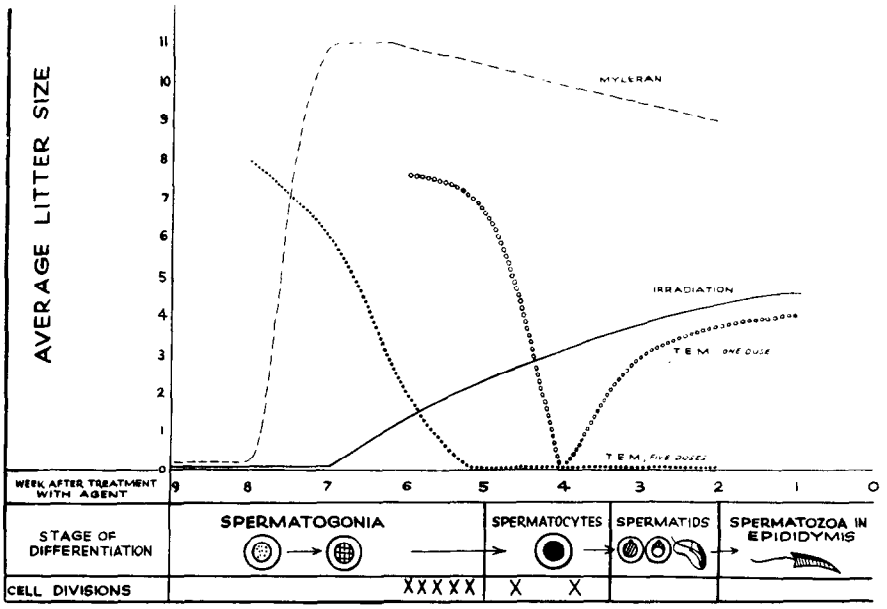

FIG. 12. Effect of cytotoxic agents on fertility of male rats. Adapted from figures of Jackson[18, 19] by smoothing the curves and reversing the time-axis to show the stages at which developing sperm is sensitive to 500 r (——), Myleran, 10 mg/kg i.p. (– – –), triethylene melamine, 0·2 mg/kg i.p. (○○○) and triethylene melamine, 0·2 mg/kg i.p. × 5 (●●●).

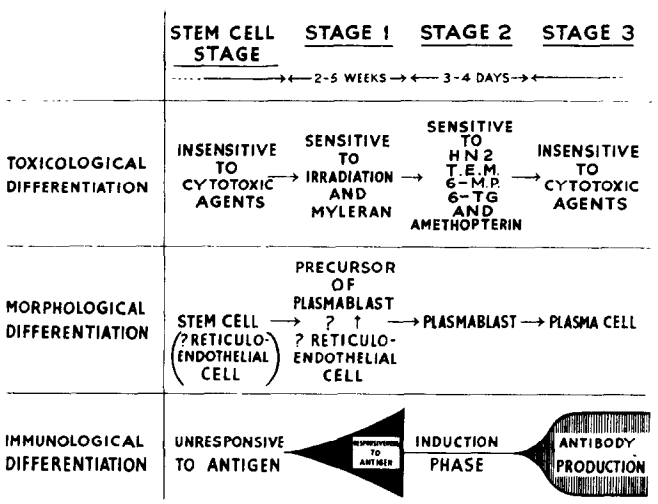

FIG. 13. Stages in the functional and morphological differentiation of immunologically competent cells and their varying susceptibility to cytotoxic agents.

to triethylene melamine after cell-division has ended. Consistent with this are the findings that spermatogonia are damaged by X-rays within a few hours of administration and that mature spermatozoa are directly damaged by triethylene melamine.[20, 21]

Similarly, gross histological changes in bone-marrow, lymphoid tissue and intestine are produced some 2–5 days sooner by nitrogen mustards than by Myleran.[22, 23] This could be explained by supposing that differentiating cells are damaged at an earlier stage by Myleran than by nitrogen mustards, so that evident damage to or depletion of the more differentiated cells that constitute the bulk of these tissues is manifest sooner with the mustards.

The hypothesis to be considered, then, may be represented diagramatically as in Fig. 13. Immunologically competent cells are derived from precursors that are themselves unresponsive to antigen and perhaps relatively insusceptible to damage by the cytotoxic agents used here. These precursors give rise by differentiation, with or without division, to cells able to respond to antigen and susceptible to damage by class 1 agents. We may term these stage 1 cells. By analogy with other differentiating systems, responsiveness to antigen may be envisaged as being slight initially and increasing with progressive differentiation. If the cells receive an appropriate antigenic stimulus during this stage they undergo a further rapid differentiation, lose their sensitivity to class 1 agents and become sensitive to class 2 agents. This is stage 2 or the induction period. Eventually they become fully differentiated (stage 3), produce antibody and become insensitive to agents of both classes.

It is likely that sensitivity to irradiation begins not many days before the stage at which the cells first become able to respond to antigen. If radiosensitivity began much before this there would be a corresponding delay before immunological responsiveness began to recover in irradiated animals. In fact recovery begins quickly, although it takes some weeks to complete.[11, 15]

It remains to consider what known effects of the cytotoxic agents could be responsible for impairing the functions of the cells concerned in the immune response. The effects it seems appropriate to consider in the first place are inhibition of phagocytosis, of synthesis of DNA, RNA and protein and inhibition of mitosis in, and histologically visible damage to, lymphoid tissue. It appears advisable to limit consideration to changes produced *in vivo* by doses in the immunologically effective range.

It is unlikely that irradiation, in the doses used in these experiments, acts by interfering with uptake or breakdown of antigen,[24, 25] and Myleran does not interfere with phagocytosis.[26] Clearly, suppression of phagocytosis by class 2 agents could not be the means by which their effect was exerted for they are most effective at a time when phagocytosis of particulate antigens, such as that used here, is complete.

Irradiation inhibits synthesis of DNA and nuclear RNA under some circumstances but has little effect, and sometimes none, on production of cytoplasmic or total-cell RNA (the recent literature is reviewed by Gouttier[27]). Nitrogen mustard depresses DNA synthesis; inhibition of RNA synthesis is usually less marked and sometimes absent.[28–30] Similar findings have been reported for 6-mercaptopurine[31, 32] and amethopterin.[33, 34] Equal depression of DNA and RNA synthesis was reported in one experiment on thioguanine.[35]

These agents do not affect protein synthesis *in vivo* sufficiently to account for their ability to inhibit production of antibody.[34, 36]

The importance of these various effects and the fact that they are produced *in vivo* by immunologically effective amounts of these agents strongly suggest that they play some part in suppressing the immune response. Nevertheless, this presumption should not be allowed to obscure the lack of convincing experimental evidence on this point. The fact that these agents inhibit synthesis of DNA and RNA or of particular fractions of RNA is not in itself proof that it is by these mechanisms that they suppress the immune response.

Irradiation, nitrogen mustard and triethylene melamine inhibit mitosis in lymphoid tissue and rapidly destroy lymphocytes.[37–40] Gross histological damage of lymphoid tissue is sometimes put forward as the explanation of the immunological effects of these agents. However, the timing of such changes is similar with all three agents and does not lead one to expect that the time of most effective administration of the alkylating agents should be so different from that of X-rays. Moreover, this explanation cannot hold for 6-mercaptopurine, thioguanine, amethopterin or Myleran. The histological changes caused by these substances in lymphoid tissue are slight; they may inhibit mitosis in various other tissues but this has not yet been reported as occurring in lymphoid tissue.[22, 41–44]

There can be little doubt that the present inability convincingly to incriminate particular biochemical or morphological lesions as being responsible for the immunological effects of these agents is due largely to lack of adequate knowledge about their modes of action. Much of the information available must be regarded as preliminary and, even with clearly defined biochemical effects, it has yet to be determined whether these or cell-death and changes in cell-population are primary.[27, 45, 46]

Should detailed study of antibody-forming tissue reveal metabolic processes, cell-types or cell-stages selectively affected by irradiation and Myleran and other processes or cells selectively affected by the other agents used, there would be a strong presumption that the difference in action of these agents on the immune response is due to this selectivity. In this connexion, it may be significant that the mustards and ethylene-imines produce considerable cross-linking of DNA in intact cells, whereas X-rays and Myleran do not do so to a notable extent.[47, 48] It is possible, therefore that DNA in cells at relatively late stages of differentiation is highly susceptible to cross-linking by mustards and ethylene-imines *in vivo*, whereas that in less differentiated cells is less susceptible.

Since irradiation and Myleran do not significantly cross-link DNA in intact cells, it must be presumed that they damage cells in some other way. The possibility that relatively undifferentiated cells are particularly sensitive to these agents, whereas more differentiated cells are not, may afford a clue to the way in which they act.

These experiments also have a bearing on current work on the homograft response and attempts to inhibit it by treatment with cytotoxic agents. It has generally been assumed that depression of the immune response to a graft is best effected by giving repeated doses of cytotoxic agent and that treatment of the recipient should commence before grafting is carried out. The findings reported here suggest that these assumptions may be unjustified, that single or a few, widely spaced large doses of agent may be as effective as many small doses, while avoiding the complication of cumulative toxicity and that, for some agents, the critical time for administration may be after and not before grafting.

Acknowledgements—I am indebted to Dr. J. F. A. P. Miller of the Chester Beatty Research Institute for carrying out the irradiations, to Professor F. Bergel of the Chester Beatty Research Institute, Dr. R. S. F. Hennessey of Wellcome Research Laboratories and Dr. Ruth Porter of Lederle Laboratories for gifts of chemicals and to Dr. J. H. Humphrey and Dr. P. Alexander for most helpful discussions. Mr. P. R. Toothill examined the experimental results statistically. Technical assistance was provided by Mr. W. A. Cope, Miss C. Newbery and Mr. M. Chainey.

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