Immunology, 1963, **6**, 356.

Immunological Unresponsiveness to Protein Antigens in Rabbits Exposed to X-Irradiation or 6-Mercaptopurine Treatment

D. NACHTIGAL AND M. FELDMAN

Section of Cell Biology, Weizmann Institute of Science, Rehovoth, Israel

(*Received 3rd December* 1962)

Summary. Immune unresponsiveness to HSA and BSA was induced in adult rabbits exposed to 550 R. total-body irradiation. Immunogenic doses of antigen, 20–75 mg., were found to be tolerogenic in this system. The degree of unresponsiveness was found to be a function of the time interval between X-irradiation and the beginning of antigen administration. The unresponsive animals retained the circulating antigen in quantities detectable by the gel-diffusion technique. These results were compared with the state of unresponsiveness induced by means of 6-mercaptopurine, and similar parameters were found to be operative. Some aspects of the significance of the ratio of antigen to competent cells, in the induction of immune unresponsiveness, are discussed.

INTRODUCTION

Specific immunological unresponsiveness to defined antigens can be induced in adult animals by a number of experimental methods. Felton (1949) was the first to observe unresponsiveness in mice after treatment with large doses of pneumococcal polysaccharide, and 10 years later this phenomenon was shown to result from a central failure of antibody production (Sercarz and Coons, 1959). Similar results were obtained by Dixon and Maurer (1955), who exposed adult rabbits to large doses of antigen. When X-irradiated rabbits were treated with the same large doses of antigen, the state of unresponsiveness was found to be more prolonged than in normal animals. Thus, physical methods causing depletion of the lymphoid tissues were shown to facilitate the induction of tolerance. More recently, chemical means, having perhaps a similar effect in depleting the lymphatic system, were found to be 'tolerogenic'. Schwartz and Dameshek (1959) made rabbits specifically non-responsive to protein antigen by treatment with 6-mercaptopurine. Another antimetabolite, thioguanine, was found to act in a similar way (Sterzl, 1961).

These and other experiments on the induction of immunological unresponsiveness in adult animals open a new approach to the analysis of immune tolerance. It appears that the ratio of antigen to immunologically competent cells in the adult animal is one of the factors which determine whether tolerance is induced. It can accordingly be predicted that after cellular depletion of the antibody-forming organs, lower and otherwise immunogenic doses of antigen will be capable of inducing tolerance. The experiments reported in the present paper are intended to test this and other expectations concerning unresponsiveness to protein antigens in adult rabbits. However, further consideration of the results, though they appear to verify the predictions, raises new dilemmas which are pointed out in the Discussion.

MATERIALS AND METHODS

Animals

Rabbits of both sexes, from local stock, aged 2–3 months, weighing between 2 and 3 kg. and fed standard laboratory diet, were used in the present study.

Irradiation

Total-body irradiation of 550 R. was administered from a General Electric X-ray machine (Maximar III), at 250 kVP, 15 mA. The conditions were: HVL: 1·40 mm. Cu; filtration employed: 0·5 mm. Cu + 1 mm. Al. FSD—72 cm.; field: 29 × 29 cm.; exposure dose: 28 R./min. The animals were irradiated in an open Lucite frame.

Antigens

Human serum albumin (HSA), bovine serum albumin (BSA) and human γ globulin (HGG), which had been prepared at the Plasma Fractionation Institute of the local First Aid Society according to Cohn's fractionation method, were used as antigen in this investigation. The proteins were dissolved before use in sterile saline and injected into the marginal ear vein of the rabbits in 20 or 75 mg. amounts in 1 ml. of solution.

6-*Mercaptopurine* (6-*MP*) *Treatment*

6-MP was obtained through the courtesy of Burroughs Wellcome Co., Tuckahoe, New York. One hundred mg. of the drug were dissolved in 1 ml. of 1 N NaOH. The solution was then diluted in physiological saline to the desired concentration, usually 12 mg./ml. Rabbits were injected with 75 mg. of HSA intravenously, and at the same time with 6-MP intramuscularly at a dosage of 6 mg. per kg. body weight. From then on the drug was administered daily for 13 days, at the same dose of 6 mg./kg.

Non-dissolved 6-MP, suspended directly in saline, was found to work equally well in the induction of unresponsiveness. However, it was far more toxic and caused up to 50 per cent mortality in the treated animals.

Measurement of Antibody Response

(1) Total binding antibody. Farr's labelled antigen technique, as modified by Terres and Wolins (1961), was employed for antibody estimation. The test antigen was diluted to a concentration of 100 μg./ml. Antisera binding large amounts of antigen per unit volume were diluted in appropriate amounts of pooled normal rabbit serum.

(2) Agglutinins. Stavitsky's modification (1954) of Boyden's tanned SRBC technique was employed.

(3) Precipitins. Micro gel-diffusion tests according to Crowle's template technique (1961) served to demonstrate precipitin reactions.

RESULTS

(A) IMMUNE UNRESPONSIVENESS FOLLOWING TOTAL-BODY X-IRRADIATION

In the first experiment attempting to confer specific unresponsiveness on irradiated animals, nine rabbits were injected with 75 mg. of HSA intravenously 24 hours after irradiation. Thereafter, rabbits were further challenged intravenously with 20 mg. of

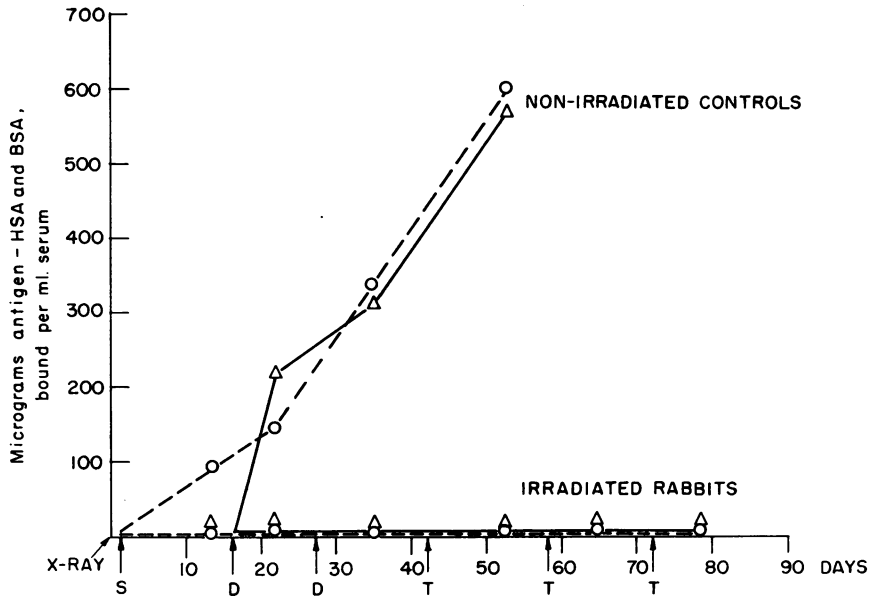

FIG. 1. The response of irradiated and non-irradiated rabbits to protein antigens. X-RAY—550 R. total-body irradiation; S—single antigen injected, 75 mg. of HSA intravenously; D—double booster, HSA and BSA, 20 mg. intravenously each; T—triple booster, HSA, BSA and HGG, 20 mg. each; △—BSA; ○—HSA.

HSA at 11–16 day intervals (Fig. 1). Starting with the first of these 'booster' injections 16 days after irradiation, 20 mg. of BSA were added; and from 6 weeks after irradiation on, the third antigen, HGG, was also injected in 20 mg. doses. Non-irradiated control rabbits underwent the same schedule of immunization as their irradiated counterparts.

The results, presented in Fig. 1, indicate that a persistent state of non-responsiveness to both HSA and BSA was found in all animals treated after irradiation (it should be noted that the injections of BSA were started 16 days after exposure to X-rays). The sera of the individual experimental animals were tested by the Farr technique. The maximal combining power for HSA throughout the test period of about 80 days was never higher than 5 μg./ml. in any of the rabbits tested; that for BSA was never above 7 μg./ml. On the other hand, four non-irradiated control rabbits, which were subjected to the same doses of antigen, showed an intense immune response to both proteins. The mean maximal binding capacities of both antigens were found to be 598 $\pm$ 59 (standard error) μg./ml. for HSA, and 566 $\pm$ 41 μg./ml. for BSA.

Table 1 shows that this suppression of antibody production to HSA and BSA in X-irradiated animals could not be attributed merely to a non-specific suppression of the immune mechanism due to general radiation injury of the antibody-forming organs. The state of unresponsiveness appeared to be specific, since for each of the three antigens tested in the same animal, the degree of unresponsiveness was a function of the time interval between irradiation and the first challenge with antigen. When the animals were tested 79 days after X-irradiation by Boyden's haemagglutination method no antibody to HSA could be found in any of the experimental rabbits. One out of nine reacted to BSA, the antigen injected for the first time 16 days after X-irradiation, whereas for HGG, which was first injected 42 days after irradiation, five out of nine animals gave positive

reactions. It should be noted that BSA was given throughout the test period in five doses, totalling 100 mg., whereas HGG was injected in three doses, totalling 60 mg. It is thus evident that immunizing doses of antigen can confer specific unresponsiveness in sub-lethally irradiated animals.

Table 2 records the results of gel-diffusion precipitin reactions with sera from this

TABLE 1

BOYDEN'S HEAMAGGLUTINATION WITH THREE PROTEIN ANTIGENS ON DAY 79 AFTER X-RAY IRRADIATION

Group of rabbits tested	*Rabbit No.*	*Test antigen*			
		No antigen	*HSA*	*BSA*	*HGG*
Irradiated test rabbits	43	0	0	0	1
	44	0	0	0	0
	45	0	0	0	0
	46	0	0	6	7
	47	0	0	0	0
	49	0	0	0	0
	50	0	0	0	2
	51	0	0	0	3
	52	0	0	0	2
Non-irradiated rabbits (controls)	53	0	8	8	8
	54	0	10	10	9
	55	0	10	10	10
	56	0	5	7	3
Normal serum	Pooled	0	0	0	0

Two-fold serial dilutions were made starting with 1 : 2. Numbers denote the last tube which showed a visible agglutination. (See Fig. 1 for schedule of immunization.)

TABLE 2

GEL-DIFFUSION TESTS WITH SERA OF IMMUNIZED, NORMAL AND X-RAY-TREATED RABBITS

Group of animals tested	*Test antigen*	*Days after irradiation*								
		10	20	30	40	50	60	70	80	90
Irradiated rabbits	HSA	0/9	0/9	0/9		0/9	0/9		0/9	
	BSA	—	0/9	0/9		0/9	0/9		0/9	
	HGG	—	—	—		0/9	0/9		0/9	
Immunization schedule	X-ray	↑ S	↑ D	↑ D		↑ T	↑ T		↑ T	
Non-irradiated control rabbits	HSA	1/4	3/4	4/4		4/4	4/4		4/4	
	BSA	—	1/4	4/4		4/4	4/4		4/4	
	HGG	—	—	—		4/4	4/4		4/4	
Immunization schedule		↑ S	↑ D	↑ D		↑ T	↑ T		↑ T	

X-ray—550 R. total-body irradiation.
S—Injection of a single protein antigen (HSA), 75 mg. intravenously.
D—Double booster—HSA and BSA, 20 mg. each intravenously.
T—Triple booster—HSA, BSA and HGG, 20 mg. each
Denominator—Number of rabbits tested.
Numerator—Number of rabbits reacting positively.

group of rabbits. It is of interest to find that the immune response, in contrast with the results obtained by the Boyden technique, was either qualitatively different (see Benedict, Brown and Ayengar, 1962), or quantitatively too weak to be reflected in the precipitin tests. None of the X-rayed experimental rabbits of this group gave any precipitins even to HGG, whereas all control animals gave a strong reaction with all three antigens.

The finding that unresponsiveness to BSA could be induced when the antigen is

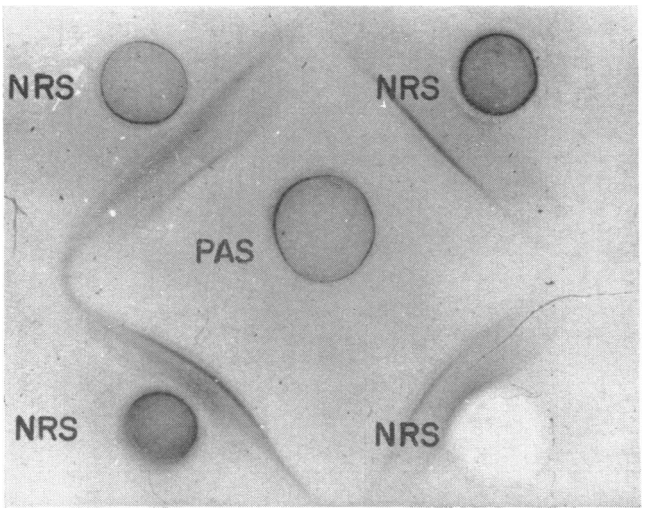

FIG. 2. PAS represents a positive antiserum from an immunized non-irradiated rabbit, which gives a positive gel-diffusion reaction with sera (NRS) from unresponsive, similarly immunized, irradiated animals. (Stained with Amido Black.)

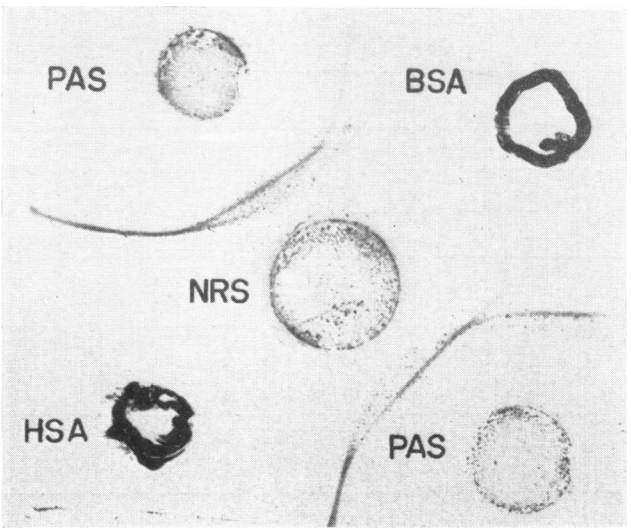

FIG. 3. Antisera, derived from immunized non-irradiated rabbits (PAS), showing a positive gel-diffusion reaction with a serum (NRS) from an animal made unresponsive to the same antigens, as well as with known antigen solutions (HSA and BSA). A pattern of identity is demonstrated by continuity of the precipitation bands formed with the antigen solutions and the NRS serum.

introduced as late as 16 days after irradiation was somewhat unexpected. We therefore tested whether this belated induction of unresponsiveness depended on the previous tolerance-inducing stimulus of HSA due to cross tolerance between closely related antigens. Animals were injected each with 20 mg. of HSA and 20 mg. of BSA, starting 15 days after X-irradiation. The rabbits thus treated remained unresponsive to both antigens. It can therefore be concluded that protein antigens can confer tolerance on X-irradiated rabbits even when injected into animals weeks after exposure to X-rays.

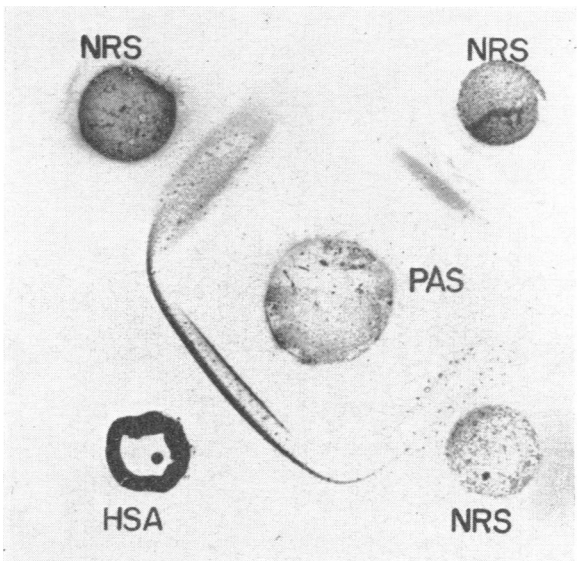

FIG. 4. Anti-HSA serum (PAS) precipitating a known solution of HSA, as well as the circulating antigen in the sera (NRS) of rabbits made unresponsive to the same antigen. Note the diffuse precipitation bands of the circulating antigen in serum as compared with the sharp band of the precipitated HSA solution.

Delayed clearance of antigen from the circulation of unresponsive animals is a characteristic feature of states of immune unresponsiveness (Dixon and Maurer, 1955; Schwartz, Stack and Dameshek, 1958; Smith, 1961), and could be found as usual in the present study. In fact, the retention of antigen in the circulation was so pronounced that it could be demonstrated by gel-diffusion tests. Invariably the sera of unresponsive rabbits gave precipitation bands when tested against antisera prepared in the corresponding non-irradiated controls (Fig. 2). These precipitin bands were identified as due to the presence of free HSA and BSA, which could be precipitated by antibody from the control rabbits. Known antigens in adjacent wells gave patterns of identity with the precipitin bands of the sera from tolerant animals (Fig. 3).

A striking and consistent characteristic of protein antigens retained in the circulation of unresponsive rabbits was that they precipitated during gel-diffusion in several relatively widespread bands, whereas antigen solutions, made up either in saline or in normal rabbit serum, gave compact precipitin bands under the same conditions (Figs. 2 and 4). It is of interest to note that the precipitin pattern seen in Fig. 5, with known HSA solution, and with the antigen retained in the unresponsive animals, is similar to the pattern of

precipitins obtained by Pope and Stevens (1958) with purified, degraded and non-degraded diphtheria toxins. This could be interpreted as evidence that the retained antigen in sera of unresponsive animals may have undergone some change, resulting in the degradation of its molecule.

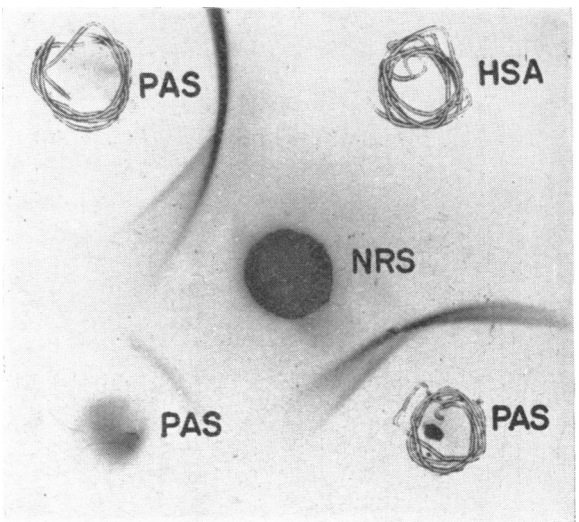

FIG. 5. Anti-HSA sera (PAS) precipitating the homologous antigen (HSA) and the circulating HSA in the serum of a rabbit (NRS) made unresponsive by irradiation. Note the splitting up of the HSA precipitation band as it approaches the NRS well.

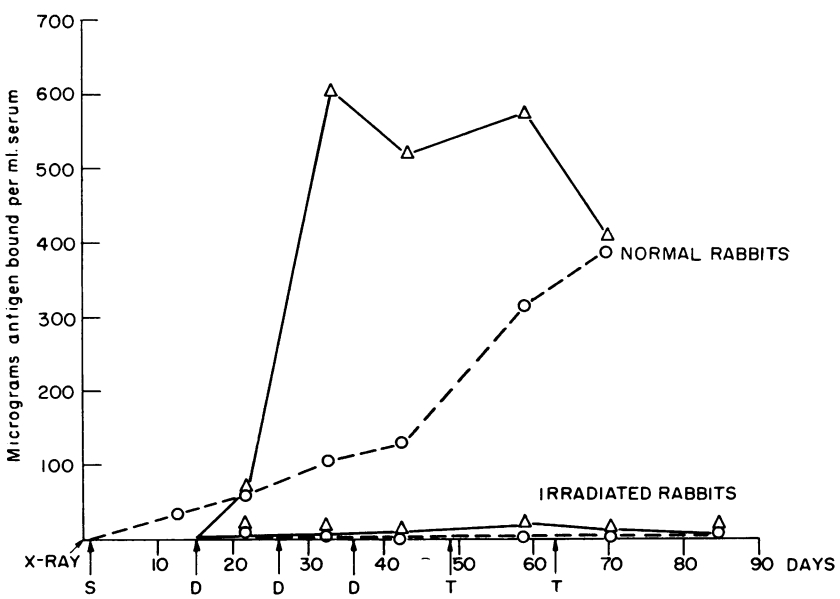

FIG. 6. The response of irradiated and non-irradiated rabbits to protein antigens. X-RAY—550 R. total-body irradiation; S—single antigen injected, 75 mg. of HSA intravenously; D—double booster, HSA and BSA, 20 mg. intravenously each; T—triple booster, HSA, BSA and HGG, 20 mg. each; △—BSA; ○—HSA.

The first experiment was then repeated with another group of five rabbits, with minor modifications in timing (and possibly also in X-ray dosage which is beyond the limits of dosimetry). The results of the first experiment were confirmed and the gradation in response to the three test antigens administered at various time intervals following irradiation were even more pronounced. Here, as in the previous group, the animals were injected with 75 mg. of HSA 24 hours after irradiation, and then with 20 mg. injections spaced at intervals of 10–15 days. No significant response to this antigen was observed within the test period of 85 days (Fig. 6). BSA, which was added to the antigen 'boosters' on day 15 following X-ray treatment, did, however, give a detectable antibody response. The binding capacity of this antibody for the homologous antigen reached a mean value of 18 ± 1·5 μg./ml. The non-irradiated controls yielded antisera with the high mean binding capacity of 578 ± 152 μg. of BSA/ml. The response to BSA of X-ray-treated animals could also

TABLE 3

TANNED CELLS AGGLUTINATION WITH THREE PROTEIN ANTIGENS AND ANTISERA OF NORMAL AND X-RAY-TREATED RABBITS

Group of rabbits tested	*Test antigen*	*Day of experiment*					
		10	20	30	40	50	60
Irradiated rabbits	HSA	—	0/5	0/5	0/5		0/5
	BSA	—	0/5	0/5	0/5		2/5
	HGG	—	—	—	—		3/5
Immunization schedule	X-ray ↑ S	↑ D	↑ D	↑ D		↑ T	↑ T
Non-irradiated control rabbits	HSA	—	4/4	4/4	4/4		4/4
	BSA	—	2/4	4/4	4/4		4/4
	HGG	—	—	—	—		4/4
Immunization schedule	↑ S	↑ D	↑ D	↑ D		↑ T	↑ T

X-ray—550 R. total-body irradiation.
S—Injection of a single protein antigen (HSA), 75 mg. intravenously.
D—Double booster—HSA and BSA, 20 mg. each intravenously.
T—Triple booster—HSA, BSA and HGG, 20 mg. each.
Denominator—Number of rabbits tested.
Numerator—Number of rabbits reacting positively.

be detected by the Boyden method (Table 3), where two out of five animals showed positive reaction, though this was much delayed (day 60) in comparison with that of the non-irradiated control animals. However, again as in the previous group, the antibody to BSA of these X-ray-treated rabbits could not be demonstrated as precipitation in the gel-diffusion test (Table 4).

The third antigen, HGG, was added to the 'booster' injections of the experimental animals 7 weeks after irradiation. This antigen elicited a good antibody response. Positive reactions were obtained by the Boyden test, and, moreover, the sera of all rabbits gave positive gel-diffusion reactions on day 70 (Table 4). This response was not significantly delayed as compared with that of untreated controls. It can thus be concluded that the degree of tolerance to protein antigens injected into X-ray-treated animals was determined,

TABLE 4

GEL-DIFFUSION TESTS WITH SERA OF IRRADIATED AND NORMAL RABBITS IMMUNIZED WITH PROTEIN ANTIGENS

Group of rabbits tested	*Test antigen*	*Day of experiment*							
		10	20	30	40	50	60	70	80
Normal control rabbits	HSA	0/4	1/4	4/4	4/4		4/4	4/4	—
	BSA	0/4	0/4	4/4	4/4		4/4	4/4	—
	HGG	—	—	—	—		2/4	4/4	—
Immunization schedule		↑ S		↑ D	↑ D	↑ D	↑ T		↑ T
Rabbits made non-reactive after irradiation	HSA	0/5	0/5	0/5	0/5		0/5	0/5	0/5
	BSA	0/5	0/5	0/5	0/5		0/5	0/5	0/5
	HGG	—	—	—	—		1/5	5/5	—
Immunization schedule		X-ray ↑ S		↑ D	↑ D	↑ D	↑ T		↑ T

X-ray—550 R. total-body irradiation.
S—Injection of a single antigen (HSA), 75 mg. intravenously.
D—Double booster—HSA and BSA, 20 mg. each intravenously.
T—Triple booster—HSA, BSA and HGG, 20 mg. each.
Denominator—Number of rabbits tested.
Numerator—Number of rabbits reacting positively.

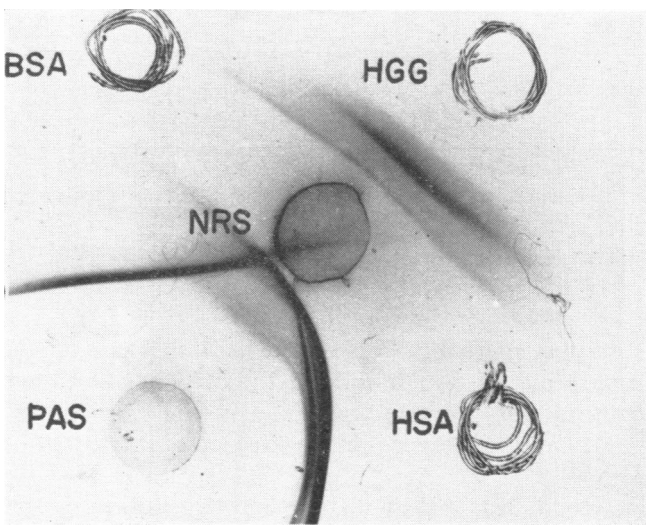

FIG. 7. A serum from a rabbit exposed to X-rays and protein antigens (NRS) does not give visible precipitation bands with two of the three administered proteins (HSA and BSA) although it precipitates the third, HGG. A serum (PAS) from a similarly treated non-irradiated animal precipitates both HSA and BSA as well as the circulating antigen in NRS serum itself. Note the identity pattern formed with the HSA precipitation band and the circulating antigen as compared with the non-identity of BSA with the antigens in NRS.

among other factors, by the time interval after irradiation at which the antigen in question was first introduced into the animal.

Tests were then carried out to find out whether the relative degree of unresponsiveness, as demonstrated in the last experiment, was reflected in the degree of retention of antigen within the circulation of the experimental rabbits. Fig. 7 shows the gel-diffusion pattern of a serum unresponsive to HSA, which was found to be partially responsive to BSA (Table 3). The anti-BSA antibody, however, could not be demonstrated by the gel-diffusion technique (see also Table 4). Thus there was no precipitation, either with HSA or with BSA, though there was a good reaction with the HGG antigen which had been

TABLE 5

GEL-DIFFUSION REACTIONS OF NORMAL AND 6-MERCAPTOPURINE-TREATED RABBITS IMMUNIZED WITH PROTEIN ANTIGENS

Group of animals tested	*Test antigen*	*Day of experiment*						
		10	20	30	40	50	60	70
Immunized and 6-mercaptopurine-treated rabbits	HSA	–	–	–		0/10	0/10	0/10
	BSA	–	–	–		2/10	7/10	10/10
	HGG	–	–	–		1/10	8/10	8/10
Immunization schedule		↑ S← 6-MP→			↑ T	↑ T	↑ T	
Normal immunized rabbits	HSA	0/4	3/4	4/4		4/4	4/4	4/4
	BSA	0/4	1/4	3/4		4/4	4/4	4/4
	HGG	–	–	–		4/4	4/4	4/4
Immunization schedule		↑ S			↑ T	↑ T	↑ T	

6-MP—Fourteen daily injections of 6-mercaptopurine, 6 mg. per kg. body weight.
S—Single injection of 75 mg. HSA intravenously.
T—Triple antigen booster—HSA, BSA and HGG, 20 mg. each, intravenously.
Denominator—Number of animals tested.
Numerator—Number of animals reacting positively.

added last to the series of immunizing injections. On the other hand, a positive antiserum of a corresponding untreated rabbit, placed in an adjoining well, which precipitated HSA and BSA, produced positive precipitin reaction with circulating antigen in the unresponsive serum. However, this precipitin band showed a pattern of identity exclusively with HSA. The BSA band cut across the well of the unresponsive serum, thus proving complete non-identity with the antigenic material of the serum of the tolerant rabbit. This suggests that an accelerated clearance of BSA from the 'partially' responsive animals took place. It thus appears that the relative degree of unresponsiveness was reflected in the degree of antigen-retention within the circulation of the test animals.

(B) IMMUNOLOGICAL UNRESPONSIVENESS INDUCED WITH 6-MERCAPTOPURINE

The state of immunological unresponsiveness of X-irradiated animals was then compared to that of animals made tolerant by means of 6-MP treatment. In one experiment, ten rabbits treated with 6-MP were subjected to a course of antigen injection similar to that of the irradiated experimental animals, except that two double boosters of HSA and

BSA were omitted. Thus, four injections of HSA, totalling 135 mg., were given, and the sera of the individual rabbits were tested by the Farr technique. The general level of total HSA binding antibody rose higher than in the irradiated rabbits. The highest mean obtained within the test period was a combining capacity for 11 ± 8 μg. of HSA/ml. Although measurable by the Farr technique, this response was too weak to be demonstrated by means of the gel-diffusion method (Table 5). However, when tested with the Boyden technique, four out of the ten rabbits gave, on day 70, positive results with HSA-coated cells. From the start of the experiment circulating HSA could be detected, in the non-reacting sera, by means of the gel-diffusion technique. Nevertheless, this reaction, which in the irradiated rabbits persisted throughout the test period, became negative in the individual 6-MP-treated animals as soon as their binding capacities for HSA rose above approximately 8 μg./ml.

Even more pronounced was the difference in the reactions of the two groups, irradiated and 6-MP-treated rabbits, to the other two test antigens, BSA and HGG. Both these antigens were first injected into the drug-treated rabbits 3 weeks after the last 6-MP injection. There was a good response to both these proteins. The anti-BSA reached, on day 70, a binding capacity for 46 ± 4 μg./ml. of homologous antigen. At the same time, both anti-BSA and anti-HGG antibodies could be demonstrated with the Boyden, as well as with the gel-diffusion, technique (Table 5), although it is evident that the response was a delayed one, as compared with untreated controls.

It seemed that in this case, too, the gradation in response to the test antigens was a function of the timing of antigen administration in relation to the drug treatment period (Schwartz *et al.*, 1958). In order to make sure that these differences did not depend on the nature of the antigens themselves, a group of twenty rabbits was injected with HSA and BSA together (80 mg. each) at the start of a routine antimetabolite series. The mean antigen-binding capacities of these rabbits, and their corresponding controls are tabulated in Table 6, and show simultaneous inhibition of antibody production against both antigens.

TABLE 6

UNRESPONSIVENESS INDUCED SIMULTANEOUSLY TO HSA AND BSA IN 6-MERCAPTOPURINE-TREATED RABBITS

Day of experiment	*Micrograms of antigen bound per millilitre serum (mean values)*			
	6-MP treated rabbits		*Untreated control rabbits*	
	HSA	*BSA*	*HSA*	*BSA*
16	0·25 ± 0·08*	1·4 ± 1·2	23·7 ± 12	81·5 ± 8
30	7·0 ± 5·6	3·9 ± 1·2	71·0 ± 4·5	82·0 ± 4·5
40	13·5 ± 1·3	6·0 ± 0·8	82 ± 4·5	82 ± 16·0

* Standard error.

DISCUSSION

The experimental induction of unresponsiveness in adult rabbits was reported by Dixon and Maurer (1955) and by Weigle and Dixon (1959). Employing tests based mainly on the rate of antigen elimination, they found that specific immunological unresponsiveness to human plasma protein and to BSA was conferred on adult animals treated with very

large doses of antigen (25 g. over 8 weeks of plasma protein, and 21 g. over 7 weeks of BSA). Non-irradiated rabbits thus treated manifested a temporary state of unresponsiveness, whereas animals which were treated with massive doses of antigen following total-body X-irradiation (400 R.) showed a persistent state of specific immunological paralysis. However, in view of the enormous antigen doses used in those experiments, and in the absence of any data supporting these observations (see Makinodan and Gengozian, 1960), further experiments along these lines were performed in the present study, with variations in two experimental parameters: (a) exposure of the adult animals to higher doses of total-body X-irradiation (550 R.), and (b) the administration of lower tolerogenic doses of antigen. The clarification of the state of unresponsiveness induced by means of 6-mercaptopurine treatment is of special interest, since Goh, Miller and Diamond (1961), trying to confirm the original observations of Schwartz and Dameshek (1959), reported negative results in an attempt to induce 'drug-induced tolerance' to protein antigens injected at a dose of 60 mg. of HSA and HGG. The successful induction of unresponsiveness to HSA, in 6-MP-treated rabbits, was detected by measurement of the rate of antigen clearance (Schwartz and Dameshek, 1959). In the present study, we applied direct tests for antibodies to the serum of the experimental animals, performed by means of the Farr, the Boyden, and gel-diffusion methods. Evidence is given that otherwise potentially immunogenic doses of antigen of 20–70 mg. protein could confer tolerance in X-rayed and 6-MP-treated animals. The level of tolerance depended on the time of first antigen administration in relation to the time of physical or chemical treatment. The tolerant animals retained the circulating antigens in quantities sufficient to be demonstrable by gel-diffusion precipitin tests.

The data reported bear on the role, during induction and maintenance of unresponsiveness, of the ratio of antigen dose to the size of the competent cell population. It is generally believed that the induction of specific immune non-reactivity to protein antigens, whether termed 'immune tolerance', 'drug-induced unresponsiveness' or 'immune paralysis', depends on the administration of a minimal tolerogenic dose of antigen which varies in quantity according to the experimental system employed. Thus, immune tolerance can be induced with very low antigen doses (0·1 mg.) administered to newborn rabbits, and the minimal dose increases with age (Smith and Bridges, 1958). On the other hand, Dixon and Maurer (1955) induced immune unresponsiveness in adult rabbits by overloading the animals with heavy doses of protein antigens, reaching levels of 20–25 g. per animal. One might argue, however, that as far as the relative concentration of antigen is concerned, both experimental systems represent a similar situation: milligrams of foreign protein injected into neonatal rabbits might overload the prospective competent cells to an extent similar to that of grams of antigen administered to adults. There is a wealth of experimental data compatible with the view that the ratio of antigen to competent cells plays a decisive role in the induction of immunological unresponsiveness.

A comparative analysis of such data seemed, until recently, to indicate that the tolerogenic dose of antigen per unit weight of the animal was greater for adult animals, and that relatively larger amounts of antigen seemed to be required to paralyse an adult organism than to confer tolerance on a newborn animal (Hanan and Oyama, 1954; Cinader and Dubert, 1955, 1956; Dixon and Maurer, 1955). Yet, in a recent study (Dresser, 1962) it has been found that, in mice, there is no difference between the relative amounts of antigen required to induce unresponsiveness in adults and that required for newborns. However, Dresser's system is a rather unusual one, since the small doses of antigen used there are not

immunogenic when administered without adjuvant. The dose of antigen seems to be the factor determining not only induction of tolerance, but also temporary immunological suppression. Thus, Nathan, Bieber, Elion and Hitchings (1961) have demonstrated that a suppression of haemagglutinins to sheep red cells in 6-MP-treated mice could be obtained only when the antigen was administered in amounts above a certain dose level. Lower doses evoked an immune response in the treated animals.

On the assumption that a minimal ratio between antigen and immune competent cells is an essential factor in inducing immune unresponsiveness, one could predict that, following depletion of such cells in the adult animal, much lower antigen doses would be required for induction. One would then expect that antigen doses which are tolerogenic in newborn rabbits would act similarly in adult rabbits which had undergone total-body X-irradiation, or treatment with 6-MP. The results reported in the present study seem to confirm this expectation.

However, further consideration of these parameters raises an important dilemma, which has recently been pointed out by Humphrey (1962). Since X-irradiation or 6-MP treatment reduce only the number of immune competent cells, but have no significant influence on the general body weight or blood volume, the extracellular antigen concentration remains the same in treated and untreated rabbits. Each single competent cell is acted upon by the same concentration of antigen molecules in treated and in non-treated adult animals. This makes the significance of the antigen-competent cell ratio difficult to explain in purely quantitative terms. The explanation might possibly rest on the assumption that the physical or chemical treatment not only depletes the number of cells but also induces, following this depletion, the appearance of cells at stages particularly susceptible to induction of unresponsiveness.

ACKNOWLEDGMENTS

This work was supported by Grant No. C-6165 and agreement No. 335105 from the National Institutes of Health, United States Public Health Service.

We are grateful to Messrs. Z. Kornbaum and S. Leib for their skilful technical assistance.

REFERENCES

BENEDICT, A. A., BROWN, R. J. and AYENGAR, R. (1962). 'Physical properties of antibody to bovine serum albumin as demonstrated by haemagglutination.' *J. exp. Med.*, **115**, 195.

CINADER, B. and DUBERT, J. M. (1955). 'Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin.' *Brit. J. exp. Path.*, **36**, 515.

CINADER, B. and DUBERT, J. M. (1956). 'Specific inhibition of response to purified protein antigens.' *Proc. roy. Soc.* B, **146**, 18.

CROWLE, A. J. (1961). *Immunodiffusion*, p. 204. Academic Press, New York.

DIXON, F. J. and MAURER, P. H. (1955). 'Immunologic unresponsiveness induced by protein antigens.' *J. exp. Med.*, **101**, 245.

DRESSER, D. W. (1962). 'Paralysis induced in adult mice by small quantities of protein antigen.' *Immunology*, **5**, 378.

FELTON, L. D. (1949). 'The significance of antigen in animal tissues.' *J. Immunol.*, **61**, 107.

GOH, K., MILLER, D. G. and DIAMOND, H. D. (1961). 'An investigation into the use of 6-mercaptopurine to induce immunological tolerance.' *J. Immunol.*, **86**, 606.

HANAN, R. and OYAMA, J. (1954). 'Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age.' *J. Immunol.*, **73**, 49.

HUMPHREY, J. H. (1962). Reported at Symposium on Immunological Tolerance, Paris, June 1962.

MAKINODAN, T. and GENGOZIAN, N. (1960). *Mechanisms of Antibody Formation* (ed. by M. Holub and L. Jarošková), pp. 339–53. Publishing House of Czech. Acad. Sci., Prague (Distr. Academic Press, New York).

NATHAN, H. C., BIEBER, S., ELION, G. B. and HITCHINGS, G. K. (1961). 'Detection of agents which interfere with the immune response.' *Proc. Soc. exp. Biol. (N.Y.)*, **107**, 796.

POPE, C. G. and STEVENS, M. F. (1958). 'The properties of crystalline diphtheria toxin protein: its antigen composition as determined by gel-diffusion methods.' *Brit. J. exp. Path.*, **39**, 150.

SCHWARTZ, R. and DAMESHEK, W. (1959). 'Drug-induced immunological tolerance.' *Nature* (*Lond.*), **183**, 1682.

SCHWARTZ, R., STACK, J. and DAMESHEK, W. (1958). 'Effect of 6-mercaptopurine on antibody production.' *Proc. Soc. exp. Biol.* (*N.Y.*), **99**, 164.

SERCARZ, E. and COONS, A. H. (1959). 'Specific inhibition of antibody formation during immunological paralysis and unresponsiveness.' *Nature* (*Lond.*), **184**, 1080.

SMITH, R. T. (1961). 'Immunological tolerance of nonliving antigens.' *Advanc. Immunol.*, **1**, 67.

SMITH, R. T. and BRIDGES, R. A. (1958). 'Immunological unresponsiveness in rabbits produced by neonatal injections of defined antigens.' *J. exp. Med.*, **108**, 227.

STAVITSKY, A. B. (1954). 'Procedure and general applications of hemagglutination and hemagglutination–inhibition reactions with tannic acid and protein-treated red blood cells.' *J. Immunol.*, **72**, 360.

STERZL, J. (1961). 'Effect of some metabolic inhibitors on antibody formation.' *Nature* (*Lond.*), **189**, 1022.

TERRES, G. and WOLINS, W. (1961). 'Enhanced immunological sensitization of mice by the simultaneous injection of antigen and specific antiserum.' *J. Immunol.*, **86**, 361.

WEIGLE, W. O. and DIXON, F. J. (1959). 'Mechanisms of hypersensitivity.' International Symposium (ed. by J. H. Shaffer, G. A. LoGrippo and M. W. Chase), pp. 529–38. Little-Brown, Boston.