DELAYED HYPERSENSITIVITY IN THE DEVELOPMENT OF CIRCULATING ANTIBODY. THE EFFECT OF X-IRRADIATION

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Intracutaneous administration of a small quantity of soluble antigen into guinea pigs produces a characteristic delayed hypersensitivity, which is ultimately followed by the appearance of circulating antibody and Arthus type hypersensitivity (1, 2). Guinea pigs sensitized intracutaneously with 1 Lf purified diphtheria toxoid in Freund adjuvant (without mycobacteria) developed delayed hypersensitivity detectable from the 4th to 5th day after sensitization to the 11th or 12th day; after the 12th day Arthus reactions could be induced and circulating antibody detected (2). This sequence of events suggests that delayed hypersensitivity is a step in the formation of circulating antibody.

Delayed hypersensitivity following administration of this purified soluble antigen is characteristic in that circulating antibody cannot be detected (<0.0024 μg. antibody nitrogen) and hypersensitivity can be transferred to normal guinea pigs with leucocytes from sensitized donors. Cellular infiltration into the skin test site is typically mononuclear (3), in contrast to the polymorphonuclear invasion in the Arthus phase. The presence of delayed hypersensitivity prior to production of antibody has also been demonstrated with such chemical allergens as picryl chloride and 2:4 dinitrochlorobenzene (4).

Studies on the effect of x-irradiation on production of antibodies have indicated (*a*) that x-rays in doses just sublethal to the host inhibited the formation of antibody when the x-rays were applied at the beginning of immunization, and (*b*) that small doses did not inhibit but actually stimulated antibody production (5). Presumably, large x-ray doses reduced anaphylactic shock because production of circulating antibody was inhibited (6). The influence of large or small doses of x-rays on delayed hypersensitivity and on the subsequent Arthus reactions has not been reported.

The present paper describes experiments indicating that total body irradiation of guinea pigs 18 to 24 hours before intradermal administration of soluble

antigen has no apparent inhibitory influence on delayed hypersensitivity but on the contrary actually results in an extension of the period during which it can be demonstrated. The appearance of circulating antibody is delayed, and the amount is reduced depending on the quantity of irradiation. The period of time following sensitization at which typical Arthus reactions are demonstrable is also delayed.

Materials and Methods

Animals.—Guinea pigs were of the Hartley strain and weighed 400 to 460 gm.

Antigen.—Purified diphtheria toxoid (KP59A) was obtained through the courtesy of Dr. James A. McComb, Biologic Laboratories, Massachusetts Department of Health, Boston. Highly purified toxoid (7, 8) was obtained from Dr. C. G. Pope, the Wellcome Research Laboratories, Beckenham, Kent, England. To reduce surface denaturation, antigens for skin testing were diluted in physiologic saline containing 1 per cent normal guinea pig serum.

Sensitization.—Antigen was dissolved in physiologic saline plus 1 per cent guinea pig serum and emulsified with an equal volume of Freund adjuvant (Difco), without mycobacteria. Guinea pigs were sensitized with 1 Lf of this oil-water emulsion by injection of 0.5 ml. intracutaneously into the digits of the feet.

Skin Tests.—Guinea pigs were tested intradermally with 0.1 ml. of purified toxoid containing 10 Lf/ml. Reactions were observed and diameters of areas of induration measured at intervals for the first 4 to 6 hours after injection and at 18 to 24 hours.

Passive Transfer of Hypersensitivity.—Popliteal, inguinal, and axillary lymph nodes were excised from 8 to 10 sensitized guinea pigs and suspended in cold Tyrode's solution. The nodes were minced and squeezed through a small garlic press, and the resulting cell suspensions washed by centrifugation 2 to 3 times in 10 to 12 ml. cold Tyrode's solution. The cells were then resuspended, counted with the aid of a Levy hemocytometer, and injected intraperitoneally into normal guinea pigs. About 48 hours later, the recipient guinea pigs were tested intradermally with 1 Lf toxoid.

X-Ray.—The animals were exposed to total body x-irradiation varying from 50 to 300 r at 250 kv., 15 ma., at 50 cm. distance, with 1 mm. Cu–1 mm. Al added filter, and with HVL (half-value layer) 1.0 mm. Cu. Except when stated, guinea pigs were exposed to radiation in the afternoon, and the following morning (about 18 hours later) sensitized with 1 Lf toxoid in Freund adjuvant. Animals were skin-tested and bled at frequent intervals, but none were bled or skin-tested more than once. The blood was used for antitoxin determination, total leucocyte count, total erythrocyte count, and differential determination of leucocytes. Each guinea pig was examined carefully for delayed or Arthus type of hypersensitivity. Non-irradiated animals were included as controls in all the experiments. An exposure of 300 r was lethal to about 50 per cent of the guinea pigs, with most of the deaths occurring 8 to 12 days after irradiation.

RESULTS

Influence of 300 r X-Irradiation on Development of Delayed and Arthus Type Hypersensitivities.—On the 4th day after sensitization, one of 4 irradiated and none of 4 control guinea pigs showed evidence of hypersensitivity as determined by an intracutaneous skin test with 1 Lf toxoid (Table I). No circulating antitoxin could be detected by the rabbit intracutaneous test (9), which can detect 0.001 units antitoxin per ml., equivalent to 0.0024 μg. antitoxin N/ml.

By the 6th day, both control and irradiated animals developed delayed

TABLE I

Type and Severity of Allergic Response in Control and Irradiated (300 r) Guinea Pigs after Intradermal Sensitization with 1 Lf Diphtheria Toxoid

Time after sensitization	Irradiated		Controls	
	Arthus	Delayed	Arthus	Delayed
days				
0	0	0	0	0
	0	0	0	0
4	0	4 x 4	0	4 x 4
	0	5 x 5	0	4 x 4
	0	5 x 5	0	6 x 6
	0	12 x 14	0	7 x 7
6	0	15 x 15	0	14 x 16
	0	15 x 16	0	15 x 17
8	0	13 x 15	0	9 x 8
	0	15 x 16	0	15 x 15
	0	17 x 17	0	17 x 18
11	0	0	0	9 x 10
	0	2 x 3	3 x 3	10 x 10
	0	5 x 5	10 x 10	20 x 20
	0	6 x 7	18 x 19	25 x 24
13	0	15 x 15	8 x 9	20 x 20
	0	16 x 19	20 x 22	12 x 12
14	0	0	13 x 13	20 x 20
			15 x 16	21 x 22
15	0	0	13 x 14	9 x 10
	0	18 x 19	14 x 14	11 x 12
17	0	15 x 16	17 x 18	0
	0	22 x 22	18 x 18	6 x 6
19	0	10 x 10	16 x 17	18 x 19
	0	11 x 12	17 x 18	10 x 10
21	12 x 13	13 x 14	12 x 13	13 x 15
	15 x 15	18 x 20	13 x 13	15 x 15
26	15 x 17	20 x 20	14 x 15	11 x 17
	15 x 16	10 x 11	15 x 16	11 x 11

Note: Since non-specific inflammation about 5 x 5 mm. in diameter can occur after intradermal injection of diphtheria toxoid, only reactions 10 x 10 mm. in diameter or greater are considered positive.

hypersensitivity. In control animals, delayed responses were superseded by Arthus reactions on the 11th to 13th day. In irradiated animals, however, delayed reactions persisted through the 19th day, after which Arthus responses could be elicited and circulating antibody demonstrated. From about the 11th to the 15th day, when the leucocyte count was lowest (Fig. 1), some irradiated animals either failed to respond to skin testing or showed subnormal delayed

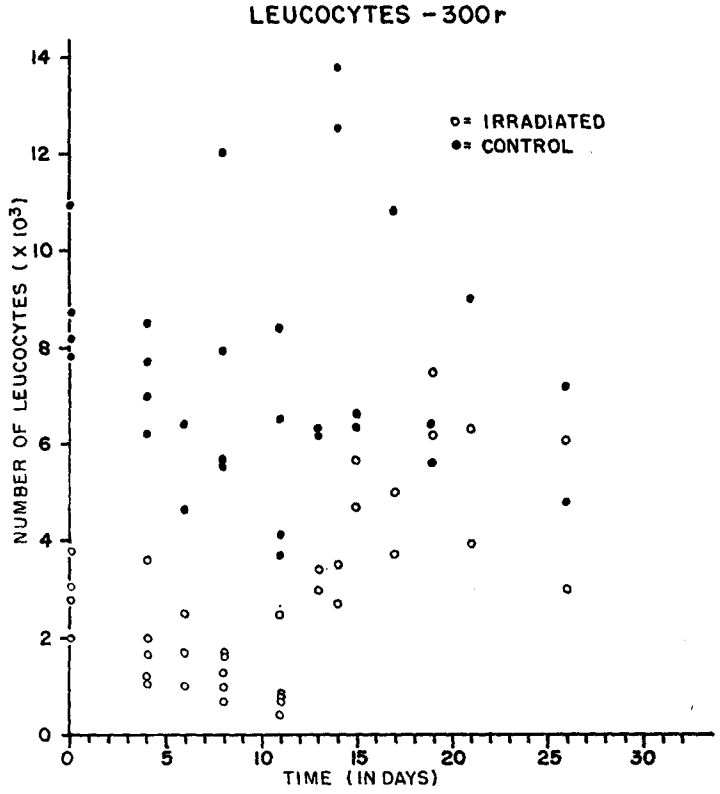

FIG. 1. Influence of 300 r total body radiation on number of leucocytes per cmm. of guinea pig blood.

reactions. Thus, x-irradiation prior to sensitization resulted in extension of the period of delayed hypersensitivity.

Irradiation produced an immediate decline in the number of circulating leucocytes, which persisted for about 21 days. During this time, delayed hypersensitivity could be demonstrated, but no circulating antibody could be detected by the rabbit intracutaneous test. Sixteen of 22 irradiated guinea pigs examined on or before the 12th day following exposure to x-ray had 2000 or less leucocytes per cmm. of blood, whereas control animals had 6000 to 8000

(Fig. 1). After the 12th day, the number of leucocytes in irradiated animals increased rapidly. Also, the differential count in the exposed animals indicated a striking preponderance of lymphocytes, with the percentage of these cells

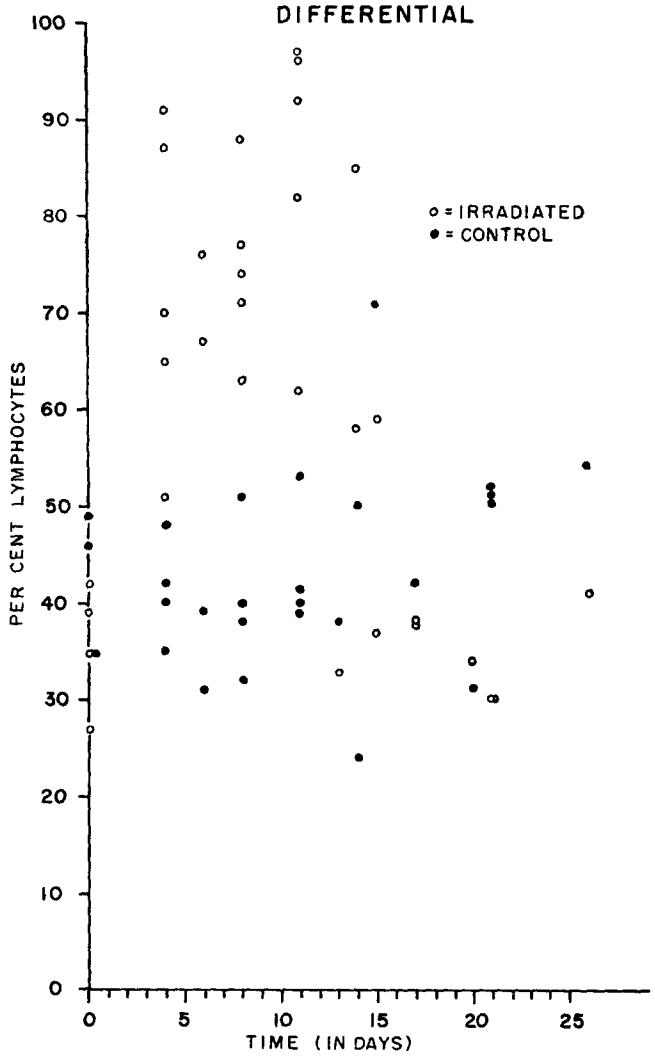

FIG. 2. Influence of 300 r total body radiation on differential leucocyte count.

increasing from about 40 per cent in the controls to over 90 per cent in some of the irradiated animals (Fig. 2). This reversal of the lymphocyte-polymorphonuclear leucocyte ratio still did not produce a normal number of lymphocytic cells. In spite of the small numbers of lymphocytes, delayed hypersensitivity

seemed to develop in a normal manner. The number of erythrocytes was also reduced by irradiation, reached its minimum about the 11th day after exposure of the animals to x-ray, and returned to normal about the 21st day (Fig. 3).

Throughout the period of delayed hypersensitivity in both irradiated and control guinea pigs, antitoxin could not be detected by the rabbit intracutaneous test. When the irradiated animals finally developed Arthus type reactions, the intensity of the reaction seemed to be about the same as in the

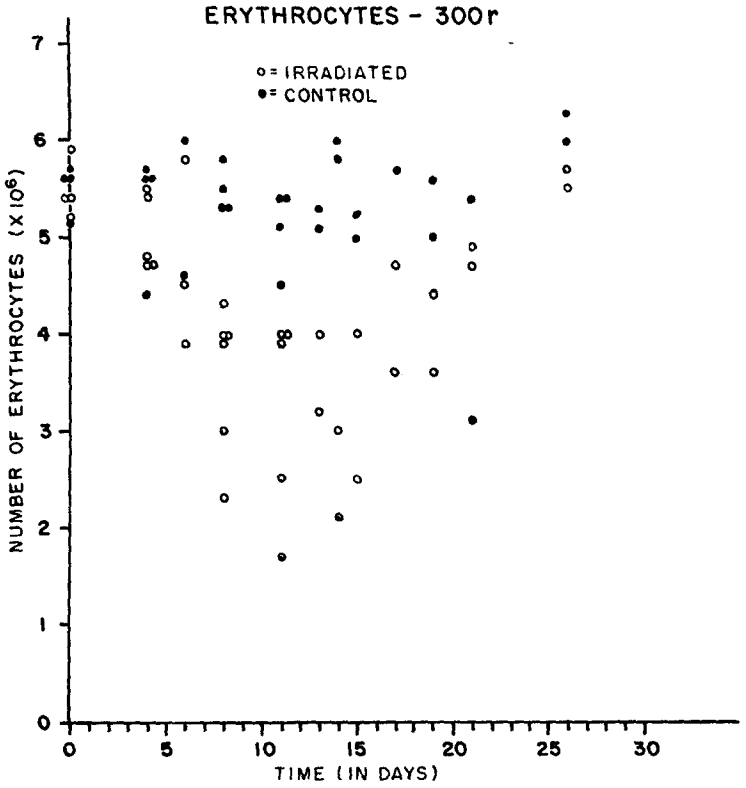

FIG. 3. Influence of 300 r total body radiation on number of erythrocytes.

controls. By that time, however, antibody titers in the controls had been increasing for over a week, and were in the range of 0.1 or more units antitoxin/ml. (Fig. 4). Circulating antibody in irradiated animals, on the contrary, had just made its appearance, and was at much lower levels. This lower amount of antibody in irradiated animals could be due to a decrease in the number of antibody-forming cells, or to actual injury of the antibody-forming mechanism.

The delayed hypersensitivity was typical as lymph node cells from sensitized guinea pigs induced hypersensitivity in normal recipients. Lymph nodes were excised 9 to 14 days after sensitization from animals that had received 300 r.

The cells that were teased out were injected intraperitoneally into normal recipients, and hypersensitivity of the delayed type was produced in 6 of 6 recipients, with induration from 13 to 20 mm. in diameter.

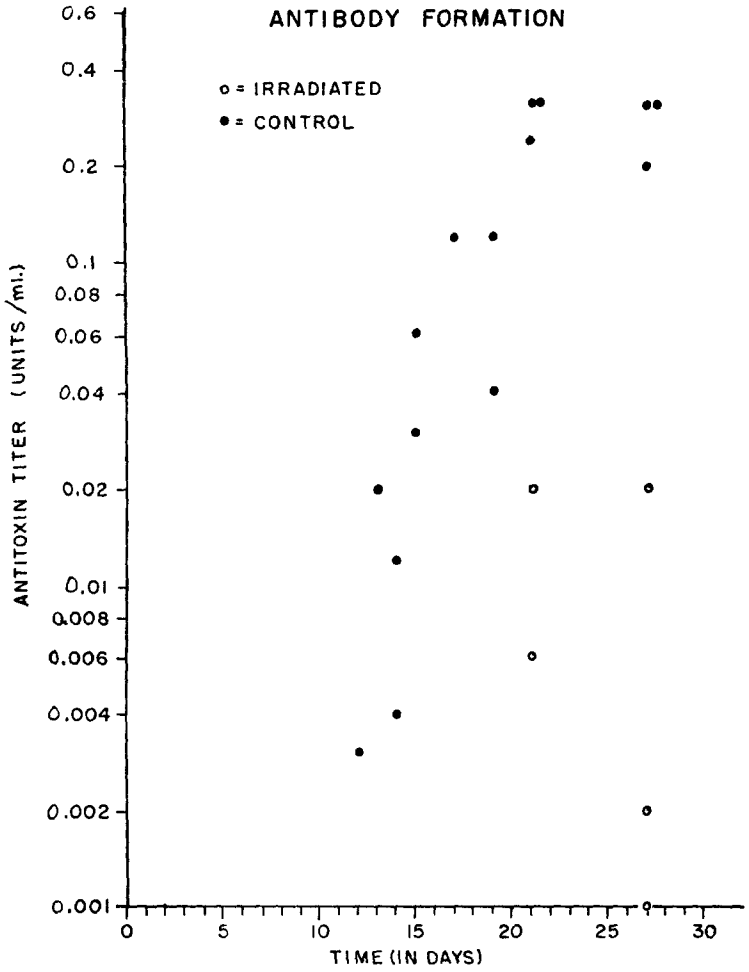

FIG. 4. Influence of 300 r total body radiation on development of circulating antibody.

Influence of Lower Doses of Irradiation (200, 100, or 50 r) on Hypersensitivity. —Since a high dose (300 r) of irradiation prolonged the period of delayed hypersensitivity prior to appearance of circulating antibody, animals were exposed to lower irradiation levels (200, 100, or 50 r) to determine the effect upon delayed hypersensitivity and circulating antibody (Tables II and III).

Guinea pigs that had received 200 r developed delayed hypersensitivity on about the 5th day. This type of reaction lasted until the 19th to 20th day, at

TABLE II

Type and Severity of Allergic Response in Control and Irradiated (200 r) Guinea Pigs after Intradermal Sensitization with 1 Lf Diphtheria Toxoid

Time after sensitization	Irradiated		Controls	
	Arthus	Delayed	Arthus	Delayed
days				
3	0	0	0	0
5	0	4 x 4	0	14 x 16
	0	16 x 17	0	15 x 17
	0	17 x 17	0	20 x 20
	0	17 x 17	0	21 x 18
	0	20 x 18		
	0	21 x 21		
	0	25 x 25		
	0	31 x 25		
7	0	18 x 22	0	9 x 9
				20 x 20
9	0	0	0	4 x 4
	0	4 x 4	0	5 x 5
	0	4 x 5		
	0	8 x 9		
	0	14 x 14		
	0	18 x 20		
12	0	12 x 12	9 x 9	18 x 18
	0	14 x 15	9 x 10	7 x 8
	0	15 x 15	16 x 15	17 x 16
	5 x 5	15 x 17	22 x 24	24 x 24
	6 x 7	18 x 18		
	0	18 x 18		
	0	19 x 20		
	0	20 x 20		
13	0	14 x 16	21 x 22	13 x 14
	0	17 x 18	23 x 24	30 x 32
14	0	10 x 10	13 x 13	19 x 20
	7 x 7	14 x 14	15 x 15	3 x 4
	6 x 6	16 x 18		
	0	17 x 18		
	0	20 x 20		
	0	20 x 20		
15	0	15 x 16	14 x 14	10 x 10
	0	21 x 21	14 x 14	12 x 12
16	0	3 x 3	15 x 16	15 x 15

TABLE II (*Continued*)

Time after sensitiization	Irradiated		Controls	
	Arthus	Delayed	Arthus	Delayed
days				
	0	7 x 8	19 x 20	23 x 24
	0	15 x 15		
	0	17 x 17		
	11 x 12	10 x 12		
19	0	13 x 13	14 x 14	19 x 19
	0	21 x 22	15 x 15	No reading
	12 x 13	20 x 21	20 x 21	11 x 12
	19 x 19	18 x 20		
20	0	15 x 16	17 x 18	10 x 10
	12 x 14	20 x 20	16 x 16	19 x 20
27	15 x 16	15 x 15	14 x 15	15 x 17
			15 x 16	11 x 11

TABLE III

Type and Severity of Allergic Response in Control and Irradiated Guinea Pigs after Intradermal Sensitization with 1 Lf Diphtheria Toxoid

Time after sensitization	100r		50r		Control	
	Irradiated		Irradiated			
	Arthus	Delayed	Arthus	Delayed	Arthus	Delayed
days						
6	0	14 x 20	0	18 x 20	0	11 x 11
	5 x 6	17 x 20	0	17 x 20	0	19 x 21
10	0	13 x 15	0	5 x 5	0	10 x 12
	0	21 x 23	0	13 x 14	0	18 x 18
12	0	5 x 7	0	5 x 5	14 x 14	8 x 9
	0	9 x 10	0	5 x 6	16 x 14	8 x 9
15	11 x 11	16 x 16	18 x 17	16 x 16	14 x 15	10 x 10
	13 x 14	16 x 16	20 x 20	14 x 14	20 x 19	16 x 16
16	15 x 15	0	0	4 x 4	13 x 13	0
	15 x 15	8 x 8	15 x 16	5 x 5	17 x 18	8 x 8
	0	16 x 16	18 x 18	5 x 5		
20	13 x 14	0	17 x 17	16 x 16	12 x 13	7 x 7
	13 x 14	8 x 8	22 x 21	16 x 16	16 x 17	7 x 7

which time it was superseded by Arthus type hypersensitivity. Duration of the delayed response was therefore about the same as in animals exposed to 300 r, and about 7 days longer than in controls.

With reduction of x-irradiation to 100 r, delayed hypersensitivity became detectable from the 5th to about the 12th day. With a further reduction in x-ray dosage to 50 r, little difference in the duration of delayed hypersensitivity was noted between irradiated and control animals. There was, therefore, a striking difference in the duration of delayed hypersensitivity as the dose of x-irradiation was decreased from 200 to 100 r. In general, increased exposure to irradiation delayed the onset of Arthus reactions and production of circulating antibody and thereby lengthened the duration of the delayed response.

Influence of Serum and Leucocytes on the Allergic Response.—The possibility exists that the delay in the appearance of circulating antibody and the apparent extension of delayed hypersensitivity may be due to depletion or damage of a non-specific serum or cell factor, such as complement.

Accordingly, 45 guinea pigs were exposed to 200 r total body radiation, and 18 hours later sensitized with 1 Lf toxoid. Fifteen of these guinea pigs received 1 ml. guinea pig complement daily intraperitoneally for 2 weeks following irradiation. Each of 15 guinea pigs of a second group was injected intraperitoneally on the 3rd, 6th, 10th, and 13th day after irradiation with a total of 1.1×10^9 leucocytes freshly isolated from normal guinea pigs. The foregoing two groups of 30 treated and irradiated guinea pigs as well as 15 irradiated guinea pigs and 15 controls were examined for early and delayed types of sensitivity for 21 days after sensitization.

No perceptible differences occurred between the treated irradiated and the untreated irradiated animals. Delayed hypersensitivity appeared on the 4th to 5th day, and was superseded by Arthus type hypersensitivity on the 19th to 21st day. In the control animals an Arthus type response was superimposed on the delayed type about the 12th day postsensitization. Thus, under the conditions of the experiment, addition of serum or leucocytes had no apparent effect on reducing the damage of x-ray to whatever regulates the rate of antibody synthesis. Factors such as complement, minerals, and the like seem not to play a major role in the delayed hypersensitivity–Arthus hypersensitivity relationship.

Influence of Temporal Relationship of Irradiation and Sensitization on Allergic Response.—In the foregoing experiments, the guinea pigs were irradiated in the afternoon, and the next morning sensitized with 1 Lf toxoid in the footpads. In order to determine the possible effect of x-ray on early stages of sensitization, the time relationship between irradiation and sensitization was varied.

When guinea pigs were x-rayed first with 300 r and within the next 24 hours sensitized with 1 Lf toxoid, the animals developed detectable delayed hypersensitivity about the 5th day and circulating antibody with Arthus type re-

sponses at about the 19th to the 21st day. Similarly, total body irradiation about the same time as sensitization resulted in an apparent prolongation of delayed hypersensitivity up to the 18th day. If the time relationship was reversed and the animals were x-rayed 18 to 24 hours *after* sensitization, the

TABLE IV

Type and Severity of Allergic Response in Control and Irradiated Guinea Pigs after Intradermal Sensitization with 1 Lf Diphtheria Toxoid

X-ray 2 hrs. after sensitization			X-ray 18 to 24 hrs. after sensitization			X-ray 72 hrs. after sensitization		
Day	Arthus	Delayed	Day	Arthus	Delayed	Day	Arthus	Delayed
5	0	7 x 8	6	0	17 x 18	5	0	5 x 5
	0	17 x 18		0	19 x 20		0	13 x 16
10	0	20 x 23	10	0	20 x 18	10	0	23 x 27
	4 x 6	22 x 25		0	23 x 20			
	10 x 12	18 x 18		7 x 8	22 x 23	12	19 x 22	22 x 25
							20 x 22	30 x 30
11	0	15 x 15	11	4 x 4	9 x 12	13	12 x 13	11 x 11
	10 x 9	22 x 22		6 x 8	12 x 13		17 x 18	17 x 17
							19 x 19	27 x 27
12	0	16 x 18	12	0	10 x 10	18	16 x 16	22 x 22
	0	17 x 19		0	17 x 19		17 x 18	14 x 14
	5 x 5	19 x 19		4 x 5	12 x 12			
14	0	15 x 16	14	12 x 12	14 x 15	20	10 x 10	15 x 16
				15 x 19	17 x 17		15 x 15	0
16	0	13 x 14						
18	15 x 15	22 x 26	17	15 x 15	18 x 20			
				15 x 15	18 x 20			
				17 x 18	14 x 13			
			20	16 x 17	20 x 22			
				18 x 18	22 x 23			

period of delayed hypersensitivity was not prolonged. The animals converted from delayed to Arthus type hypersensitivity at the same time as the controls. When the guinea pigs were irradiated with 300 r 3 days after sensitization, strong Arthus reactions could be induced on the 12th day postsensitization (Table IV).

Postponement, therefore, of irradiation for 18 to 72 hours after sensitization failed to extend the period of delayed hypersensitivity. Animals so irradiated converted to Arthus reactions at the same time as non-irradiated animals. The

non-irradiated animals, however, did develop higher antibody titers during the early days of antibody formation.

DISCUSSION

Recent studies (10–12) have demonstrated that radiation prior to antigen administration inhibits antibody production. Radiation after antigen administration has little or no effect. Three explanations for the difference between the effects of pre-antigen and post-antigen radiation have been offered: (*a*) antibody was already formed at the time of post-antigen radiation; (*b*) the full effect of radiation damage required several days; or (*c*) the first stage in antibody response was the most sensitive to radiation. Of the 3 explanations, the latter was believed to be the most logical (11).

However, investigations of the development of hypersensitivity following injection of small amounts of protein antigen suggest that delayed hypersensitivity is one of the early phases in the immune response (1, 2). In this phase circulating antibody cannot be detected, and hypersensitivity can be induced passively in recipient guinea pigs with cells from lymph nodes of sensitized donor animals. The present study shows that this phase is resistant to radiation.

Since irradiation of guinea pigs prior to injection of protein antigen prolongs the phase of delayed hypersensitivity, the suggestion may be offered that the immune response is divided into at least 3 stages. The first is radiosensitive and involves some process that determines the rate of antibody development. The second is radiation-resistant, and is expressed by delayed hypersensitivity. The third or final stage is characterized by the development and reproduction of the antibody-forming mechanism or cell, and the eventual appearance of gamma globulin. The final phase appears to be initiated within 18 hours of administration of antigen.

The initial radiosensitive phase seems to regulate the time of appearance of Arthus reactions and the onset of antibody formation. This phase lasts for relatively few hours after injection of antigen and is followed, after a short latent period, by delayed hypersensitivity. Fixation of antigen and adaptation of cells for subsequent globulin synthesis occur rapidly after administration of antigen, and are expressed days later by the appearance of specific gamma globulin. This phase is initiated within a few hours after administration of antigen, although the resulting antibody does not appear for 11 to 12 days. When the animal is irradiated shortly after sensitization, appearance of antibody is delayed for almost 10 days beyond the normal time, or about the length of time required for recovery of the leucocytes from irradiation damage.

Prolongation of the period of delayed hypersensitivity as a result of radiation may be explained by one of the following hypotheses: (*a*) The processes responsible for delayed hypersensitivity are stimulated and enhanced by radia-

tion. (*b*) Delayed hypersensitivity and antibody formation initially follow different pathways, one being either unaffected or stimulated by irradiation and the other inhibited. (*c*) Extension of the period of delayed response is only apparent, the development of circulating antibody being inhibited and preventing the Arthus reaction from masking the delayed response. The last hypothesis seems the most probable, since the delayed response is not accelerated in its appearance by radiation but can be detected on the 4th to 5th day after sensitization in both normal and irradiated animals, and since delayed hypersensitivity normally continues to be present after Arthus reactions first can be induced.

Delayed hypersensitivity is believed to be associated with lymphocytes (13, 14). Nevertheless, a sizeable decrease in the number of lymphocytes in x-rayed animals did not markedly reduce the hypersensitivity reaction. When the number eventually reached the low level of 100 to 200 leucocytes per cmm. of blood, however, no response of any kind was obtained to skin test doses of antigen. The leucocyte count was lowest at about 12 days after irradiation. When the quantity of leucocytes began to increase 2 or 3 days later, the skin reaction characteristic of delayed hypersensitivity reappeared. Apparently, the number of lymphocytic cells needed to produce a response is relatively small, with changes in the diameter of the reaction not necessarily proportional to the leucocyte number. It is possible that x-irradiation does decrease the factor responsible for delayed hypersensitivity, but since the skin test may not be a true quantitative measure of this factor, the decrease is not detectable.

Some conclusions may now be drawn on the influence of x-irradiation on delayed hypersensitivity and antibody formation. Irradiation of guinea pigs with 300 r prior to intradermal sensitization with 1 Lf diphtheria toxoid apparently does not inhibit the development of delayed hypersensitivity. The appearance of circulating antibody and Arthus type reactions is markedly delayed. When irradiation follows sensitization, delayed hypersensitivity is not inhibited in its time of appearance or in its severity. However, the time at which Arthus reactions can be induced and circulating antibody detected is the same as in control animals. Thus, there is a brief period up to 18 hours after sensitization during which the mechanism is susceptible to radiation and the rate of development of circulating antibody is influenced. Although antibody appears at the normal time, the quantity is lower in animals irradiated after sensitization, presumably because of the reduction of lymphocytic cells.

SUMMARY

The induction of delayed type of hypersensitivity to diphtheria toxoid in the guinea pig was not inhibited by total body irradiation up to 300 r in intensity. X-ray doses of 200 to 300 r administered about 18 hours before sensitization caused the period of delayed hypersensitivity to be extended to the 19th to 21st

day postsensitization in the absence of circulating antibody. X-ray doses of 50 to 100 r caused a decrease in the titer of circulating antibody, although delayed hypersensitivity lasted for a normal time. When 300 r irradiation was administered 18 hours after sensitization, delayed hypersensitivity lasted for the usual period and circulating antibody first appeared at the usual 13 to 14 days after sensitization. Introduction of normal serum or leucocytes into irradiated animals apparently did not reduce damage to the mechanism regulating the rate of antibody synthesis.

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