DELAYED HYPERSENSITIVITY*, ‡

V. The Effect of X-Irradiation on the Development of Delayed Hypersensitivity and Antibody Formation

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Plates 3 to 5

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The suppressive effect of whole body x-irradiation upon antibody formation has been extensively described in the past (1, 2). It has been shown that sufficient doses of x-ray administered *before*, but not 24 hours *after* injection of antigen usually prevent a detectable antibody response (3, 4). On the basis of such observations, Dixon *et al.* (4) have postulated two stages in the antibody response. They suggest that injection of antigen is followed by a radiosensitive adaptation phase, lasting 12 to 24 hours, followed by a radioresistant production phase.

In preliminary studies (5), it was reported that delayed-type hypersensitivity to diphtheria toxoid may develop in irradiated guinea pigs even under conditions when detectable antitoxin could not be produced. The present report describes these findings and their extension to a second species, the rabbit, and to other antigen-antibody systems.

Materials and Methods

Antigens.—Diphtheria toxoids were obtained through the courtesy of Dr. James A. McComb, Massachusetts Department of Health. KP59a containing 50L_f/ml., 1730L_f/mg. N and 1:10,000 merthiolate was used for sensitization and skin testing of guinea pigs. PT 55 containing 1400L_f/ml. and about 66 per cent specifically precipitable by antitoxin 5353AD was used as the challenging antigen for elicitation of local cutaneous anaphylaxis and for

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skin testing of rabbits. Lederle toxoid 820L_f/ml. containing 1790L_f/mg. N was used for sensitization of rabbits. Ovalbumin (2 times recrystallized) was obtained from Worthington Corp., Harrison, New Jersey.

Sensitization.—350 to 450 gm. albino guinea pigs of the Hartley strain and 1.7 to 2.3 kg. albino commerical rabbits were used. Guinea pigs were injected in all 4 foot-pads with a total of 3 μg. in 0.5 ml. of either toxoid or ovalbumin emulsified in equal volumes of saline and complete Freund's adjuvant (8.5 parts bayol F, 1.5 parts arlacel A, and 2 mg./ml. *Mycobacterium butyricum*). Rabbits were similarly sensitized except that 1 mg. of protein and a total volume of 1 ml. were injected. For tuberculin sensitization, guinea pigs were injected with 1 mg. of heat-killed, lyophilized *Mycobacterium tuberculosis* (strain H37Rv) suspended in 0.5 ml. of emulsion containing equal parts of saline and incomplete adjuvant (8.5 parts bayol F and 1.5 parts arlacel A). Rabbits were similarly sensitized with 1 ml. of this suspension.

Skin Testing.—Guinea pigs were injected intradermally with 0.1 ml. containing 3 μg. of toxoid or ovalbumin and rabbits with 0.3 ml. containing 100 μg. of either antigen. For tuberculin skin testing 0.1 ml. of 1:80 old tuberculin was used (New York City Department of Health, Lot No. 171). Reactions were examined at 2, 4, and 24 hours. The diameter of the erythema which usually corresponded to the area of induration was measured. Sections taken for histologic study were fixed in Carnoy's formalin for 1 hour.

Antibody.—Serum was obtained from guinea pigs by bleeding from the retro-orbital space with a capillary pipette. Rabbits were bled from the marginal ear vein. Antitoxin content of serum was determined by toxin neutralization in the skin of rabbits (6) and serum antibody was also searched for by passive cutaneous anaphylaxis according to the method of Ovary (7). For the latter test, 3 mg. of toxoid with Evans blue dye was used for the intravenous challenge 5 hours after intradermal injection of the test antiserum. Active cutaneous anaphylaxis (7) was performed by injecting 20 μg. toxoid or ovalbumin intradermally, and simultaneously, 0.25 ml. of 2 per cent Evans blue dye intravenously. The sites of intradermal injection were examined at 20 minutes for accumulation of dye. Agar diffusion was performed by the method of Preer (8), and quantitative precipitation by the method of Gitlin (9).

Recording of Temperature.—Guinea pigs were injected with 3 mg. of antigen intraperitoneally and their temperatures were then determined as previously described (10).

Blood Counts.—The white count was determined in a Levy hemocytometer. A smear was made with each count and 100 cells were classified for differential count. Microhematocrit readings were determined in a capillary tube sealed at both ends.

X-Irradiation.—Whole body x-irradiation was administered by a 220KV Picker x-ray machine at a distance of 75 cm. from the tube for guinea pigs, and 83 or 60 cm. for rabbits. With the machine operating at 220 kv. and 20 ma., and using a filter of 0.5 mm. copper and 1 mm. of aluminum, 25 to 40 r per minute were delivered as a midphantom dose as measured by a Victoreen ionization meter on a revolving platform. Guinea pigs were irradiated through their ventral surface; rabbits received half their x-ray dose from each side to ensure uniform absorption of irradiation throughout the animal's body. The rabbits were anesthetized with nembutal (Abbott Laboratories, North Chicago) before irradiation. Rabbits receiving 800 r received injections of 0.1 ml. of combiotic (Chas. Pfizer & Company, Inc; Brooklyn) on alternate days following irradiation.

RESULTS

Effect of X-Irradiation on Antibody Formation in the Guinea Pig.—Groups of guinea pigs received 200 r either before or after immunization with diphtheria toxoid (To). One week after immunization, guinea pigs were skin tested with 3 μg. To, and 2 weeks after immunization, serum was drawn for antibody

determinations. The serum was examined for its capacity to neutralize diphtheria toxin by rabbit skin technique which can detect extremely minute amounts of circulating antitoxin (0.0025 μg. antitoxin N/ml). To test for antibodies against non-toxigenic diphtherial proteins (*not* detected by toxin neutralization), the same serum was examined by the passive cutaneous anaphylaxis technique (PCA). The challenging antigen consisted of 3 mg. of a relatively crude To to ensure the presence of sufficient amounts of impurities to detect antibody directed against a minor component of To, if present. In addition, 18 hours after serum was obtained, active cutaneous anaphylaxis (ACA) was

TABLE I

Antibody Formation in X-Irradiated Guinea Pigs

No. of animals x-irradiated*	No. of survivors	X-irradiation, before (−) or after (+) sensitization	Antibody detectable by		
			Toxin neutralization	Passive cutaneous anaphylaxis	Active cutaneous anaphylaxis
		hrs.			
Not done	16	—	16/16	16/16	16/16
74	31	−24	1/31	2/31	13/28
24	6	−48	0/6	0/6	3/6
24	10	+48	8/10	8/10	8/10

* Guinea pigs received 200 r whole body x-irradiation before or after foot-pad injection of 3 μg. To in complete Freund's adjuvant (including killed mycobacteria).

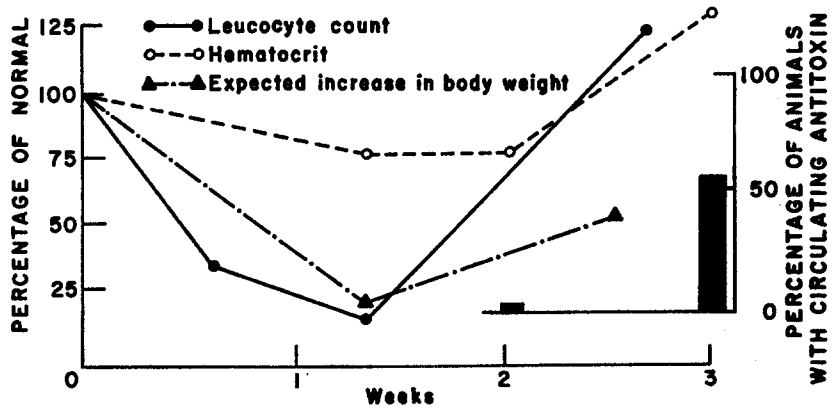
performed upon each animal in order to detect gamma globulin antibody that had become "fixed" to sites in the skin.[1]

As shown in Table I, all of 16 unirradiated control animals had antibody detectable by all 3 tests. The antitoxin content of their pooled serum was 0.25 units (250 times the minimum that can be detected). In contrast, almost all guinea pigs irradiated *before* immunization had no detectable circulating antibody. However, in 13 of 28 such animals it was possible to detect traces of antibody by ACA. The specificity of the ACA reaction was firmly established in the following manner: Both To and ovalbumin (Ea) had been injected intradermally into all the experimental animals (shown in Table I), as well as a control group of irradiated animals that had been immunized with Ea. In

[1] There is considerable evidence to indicate that the elicitation of passive anaphylaxis (local or systemic) depends upon the presence of conventional antibody that has become "fixed" to sites in the tissues, and not upon free, circulating antibody (11, 7). However, there has not been a universal acceptance of this concept (12). Furthermore, although anaphylaxis (local or systemic) in the actively sensitized animal appears to be an analogous immune reaction to passive anaphylaxis, this also has not yet been proven. In this paper, we will use the term "fixation" of antibody according to the prevalent concept, but with the foregoing qualifications in mind.

each group, dye accumulation of over 10 mm. diameter only occurred at the site of specific antigen challenge. It was of interest that an even higher percentage of such Ea "controls" showed antibody. Increasing the interval between irradiation and subsequent immunization to 48 hours did not eliminate this small amount of antibody. When To was injected 48 hours *after* irradiation, 8 of 10 animals formed circulating antibody. The effect of 300 r could not be studied, since only 1 of 24 animals survived this dose of irradiation for 2 weeks.

Return of Antibody Formation.—The sera of 24 guinea pigs x-irradiated 24 hours before immunization with To (see Table I) were examined 3 weeks later for circulating antitoxin.



TEXT-FIG. 1. Circulating antitoxin, hematopoiesis and weight gain in x-irradiated guinea pigs. Circulating antitoxin was measured in 24 guinea pigs irradiated before To immunization (see Table I). Each point in the figure represents the average determinations from 6 such animals. The differential white count revealed a change in percentage of lymphocytes from a control value of 63 to 92 at 10 days.

As can be seen in Text-fig. 1, circulating antitoxin was detected in many of the x-irradiated animals at this time. This suggested that the animals were recovering from the effects of irradiation and had been restimulated by persisting antigen. Since the initial immunization was administered in complete Freund's adjuvant, sufficient antigen for reimmunization probably remained.

In order to determine the time of recovery of other functions depressed by x-ray, six irradiated animals were weighed and bled at approximately weekly intervals for determination of hematocrit, leucocyte count, and differential count.

The findings shown in Text-fig. 1 that capacity to gain weight and hematopoiesis had begun to recover at 2 weeks lend support to the suggestion that the appearance of antibody was due to the recovery of the immune mechanism.

Delayed Skin Reactivity.—The guinea pigs described in Table I were skin-tested with 3 μg. of To 1 week after sensitization. Table II shows that all

unirradiated animals had reactions which averaged 20 mm. in diameter. 7 of 27 animals irradiated 24 hours before immunization failed to show a skin reaction, and the average diameter of the positive reactions was 16 mm. Whether or not such animals produced detectable serum antibody did not appear to influence the results. Animals irradiated after immunization all showed delayed reactions whose average diameter was 22 mm.

Biopsies were taken from 6 delayed reactions occurring in animals that received 200 r before sensitization, and from 3 reactions in unirradiated control animals. The microscopic appearance of the reactions in both groups was qualitatively similar, however, the extent of the cellular reaction was considerably reduced in the irradiated animals. Figs. 1 and 2 show a representative

TABLE II

The Development of Delayed-Type Skin Reactivity in X-Irradiated Guinea Pigs

X-irradiation, before (−) or after (+) sensitization	Antibody detectable at 2 wks.	No. of animals showing delayed skin reactions at 1 wk.*	Average diameter of skin reactions
hrs.			*mm.*
0	+	16/16	20
−24	0‡	10/13	16
−24	+	10/14	16
+48	+	10/10	22

* All animals were sensitized to To and were skin-tested 1 week later with 3 μg. To. The reactions were read at 24 hours.

‡ Those animals that did not show antibody by active cutaneous anaphylaxis 1 week after the skin test.

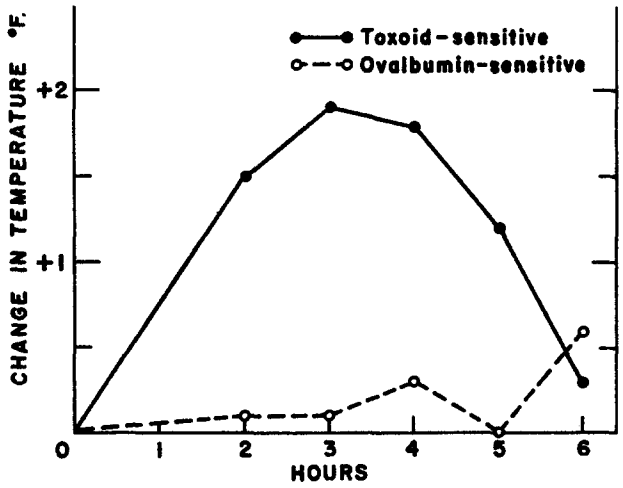
reaction in an irradiated animal. There is an infiltration of cells predominantly of the lymphoid and histiocytic type in the dermis and subcutaneous tissue. The inflammatory reaction is most marked in the vicinity of venules thus producing a lesion closely resembling that seen in "tuberculin-type" skin reactions (13).

The capacity, therefore, to develop delayed skin reactivity usually persists in guinea pigs receiving 200 r before immunization even when detectable serum antibody is not produced. The intensity of delayed skin reactivity in such animals is definitely decreased, however, as evidenced by the following findings: (*a*) One out of every 4 irradiated animals, in contrast to unirradiated controls, showed no visible reaction after specific challenge. (*b*) The reactions in irradiated animals were less indurated and their average diameter was slightly smaller than in controls. This partial suppression of delayed skin reactivity may represent another effect of 200 r on the immune system or may be a non-specific effect. Guinea pigs 1 week after receiving 200 r were usually thin, weak, and ill looking, and all suffered from a severe leucopenia including a lymphopenia.

Specific Febrile Response.—Guinea pigs with delayed-type hypersensitivity

to protein antigens develop a characteristic febrile response after injection of a large amount of specific antigen. This specific fever does not occur in guinea pigs passively sensitized with small amounts of conventional antibody, and therefore appears to be due to the interaction of antigen and delayed-type hypersensitive cells (10).

In order to see if this response persisted after irradiation, 16 animals received 200 r 24 hours before one-half were sensitized to To and one-half to Ea. 10 days later all were injected intraperitoneally with 4 mg. To and their temperatures recorded.



TEXT-FIG. 2. Specific febrile response in x-irradiated guinea pigs. 5 To- and 5 Ea-sensitive animals were challenged intraperitoneally with 4 mg. To. The average temperature elevations of both groups are shown above.

Text-fig. 2 shows that irradiated guinea pigs showed a specific febrile response of similar magnitude to that previously described in unirradiated sensitive animals.

Effect of X-Irradiation on Delayed Hypersensitivity and Antibody Formation in Rabbits.—Groups of rabbits received 400 r, 48 hours before foot-pad injection of 1 mg. of To or Ea in complete Freund's adjuvant. In others, 800 r was administered in 2 equal doses, 48 and 24 hours before immunization. Skin testing was done 12 days after immunization, except for the 800 r group which were skin-tested with controls, 7 and 10 days after immunization.

As shown in Table III, delayed skin reactivity to To developed in all rabbits receiving 400 r although such rabbits did not produce detectable serum antitoxin. 800 r before immunization abolished the development of delayed skin reactivity to To. Unirradiated immunized animals usually showed large Arthus-

TABLE III

Delayed-Type Skin Reactivity and Antibody Formation in X-Irradiated Rabbits

Immunization	X-irradiation dose	Delayed skin reactions to 100 μg. specific antigen	Serum antibody*
		mm.	
To	None	60 × 45	1.5 units antitoxin/ml.
		45 × 40	1.0
		40 × 35	0.1
		35 × 35	0.5
		40 × 40‡	2.0
	400	25 × 25	<0.001 units antitoxin/ml.
		25 × 25	<0.001
		40 × 35‡	<0.001
		30 × 35‡	<0.001
		55 × 50‡	<0.001
	800§	0	Not done
		0	
		0	
		0	
		0	
Ea	None‖	20 × 20	+
		50 × 40	+
		30 × 35	+
		40 × 40	+
		45 × 45	+
	400	35 × 30	0
		35 × 32	+¶
		0	0
		0	0
		30 × 35	0
		0	0
		0	0
	800§	0	Not done
		0	

* Antibody to To was measured in units of antitoxin/ml. Individual sera were examined for anti-Ea by both double Preer agar diffusion and PCA.

‡ Received an additional 1 mg. of To intravenously for immunization.

§ Skin-tested along with a control group on the 7th day. Control group showed reactions to To and Ea averaging 37 and 40 mm. in diameter, respectively. On the 10th day x-irradiated animals were skin-tested for the 2nd time and again showed no reactions. All other animals shown above were skin-tested 12 days after sensitization. All recorded reactions were read at 24 hours.

‖ Pooled serum of this group contained 450 μg. anti-Ea protein.

¶ Antibody detected by PCA only.

type reactions at 4 hours and the 24 hour "combined" reactions were usually more indurated than the reactions in irradiated animals. Sera from such animals had antitoxic contents ranging from 100 to 2000 times the minimum that can be detected. By 3 weeks, some of the rabbits that had received 400 r also showed Arthus-type reactions and measurable serum antitoxin (see also Text-Fig. 1).

TABLE IV

Tuberculin Hypersensitivity in X-Irradiated Rabbits and Guinea Pigs

Species	Dose of x-irradiation	Delayed skin reactions to old tuberculin*
	r	*mm.*
Rabbit	None	25 × 30
		25 × 20
		20 × 20
		0
		25 × 20
	400	30 × 30
		0
		25 × 25
		25 × 25
		0
		0
Guinea pig	None	18 × 17
		17 × 15
		13 × 13
		0
		0
		10 × 10
	200	12 × 15
		0
		0
		10 × 10
		0
		0

* 0.1 ml. of 1:80 old tuberculin was injected intradermally into rabbits 12 days after sensitization, and into guinea pigs 7 days after sensitization. Reactions were read at 24 hours.

Table III also shows that essentially analogous results were obtained using Ea except that delayed skin reactions in controls were slightly smaller and less indurated, and only one-half the animals receiving 400 r developed skin reactivity. Biopsies taken from delayed reactions in x-irradiated rabbits showed the histologic alterations typical of delayed-type reactions in unirradiated rabbits (Fig. 3).

Tuberculin Hypersensitivity.—The effect of irradiation on tuberculin hypersensitivity was studied.

Guinea pigs and rabbits were injected in all 4 foot-pads with 1 mg. killed human tubercle bacilli in incomplete adjuvant, 24 or 48 hours after irradiation, respectively. Skin testing was done 7 days after sensitization in guinea pigs, and 12 days later in rabbits.

Because of the short interval between sensitization and intradermal challenge in this experiment, the control animals were not highly sensitized. As in earlier experiments, animals that received irradiation had partial suppression of delayed skin reactivity (Table IV).

DISCUSSION

The present observations reveal an additional operational difference between the development of delayed-type hypersensitivity and antibody formation in experimental animals. Irradiated immunized animals that cannot form detectable serum antibody usually retain the capacity to exhibit the delayed type of hypersensitivity. Moreover, analogous results were obtained in a species in which delayed-type hypersensitivity is readily induced (guinea pig) and in a second species more refractory to its development (rabbit). There are at least 2 interpretations that may account for these results: (*a*) The development of the delayed type of hypersensitivity depends upon a different mechanism from that of antibody formation and is more resistant to x-irradiation. (*b*) The development of both these immune responses depends upon the same mechanism, but the delayed type of hypersensitivity is a more sensitive expression of antibody formation than other conventional serologic tests. Since there are no quantitative data on the relative sensitivity of methods for detecting serum antibody as compared to delayed-type hypersensitivity, neither explanation can be excluded at present.

In contrast to the relative radiosensitivity of the primary antibody response, the capacity to reject homografts, the specific anamnestic antibody response, and, in our studies, the delayed type of hypersensitivity are relatively radioresistant. The radioresistance of these latter immune responses might be due to a common underlying mechanism. Numerous investigators have suggested delayed-type hypersensitivity as the mechanism underlying the homotransplantation reaction (14–17) and Pappenheimer *et al.* have postulated that delayed-type hypersensitivity may similarly be responsible for the characteristic features of the specific anamnestic response (18). It is known that 800 r before secondary antigen challenge affects but does not eliminate the anamnestic response (4), but it does suppress the transplantation reaction to homologous bone marrow in at least 15 per cent of rabbits (19). Our studies have demonstrated that 800 r prior to sensitization with toxoid or ovalbumin prevented development of delayed-type skin reactions in all instances. The meaning of these results, however, is limited by the same objections raised previously; *i.e.*, the lack of information about the relative sensitivities of the test systems used.

Our results are in general agreement with those recently reported by Salvin

and Smith (20) who studied the effects of x-irradiation upon toxoid-immunized guinea pigs. The main points of difference are their findings that 200 to 300 r *completely* suppressed antibody formation *without* affecting delayed-type hypersensitivity. Since these authors tested their animals only for circulating antitoxin, they would not have detected antibody that had become bound to tissues if it were present. The depression of delayed skin reactivity that we observed was not reported by Salvin and Smith. These variant observations might be accounted for by differences in the type of guinea pig used and in animal care, factors known to be important in determining resistance to whole body x-irradiation. This possibility is suggested by the fact that the LD_{50} for the animals used by these workers was appreciably higher than in our animals.

An interesting fact to emerge from these studies is the greater sensitivity of a test for antibody which has become "fixed" to skin, compared to methods for detecting circulating antibody. This is strikingly illustrated by the results of toxoid immunization in previously irradiated guinea pigs. Antibody could not be detected in the circulation of all but a few such animals by either toxin neutralization, known to detect as little as 0.0025 μg. antitoxin N, or by passive cutaneous anaphylaxis testing, purported to detect as little as 0.003 μg. antibody N.[2] In contrast, active cutaneous anaphylaxis detected antibody in over one-third of such animals. The probable explanation for these findings is based on the known capacity of guinea pig tissues, notably skin, to efficiently bind gamma globulin (22). Presumably, if antibody production is small, antibody may be removed from the circulation by "fixation" to tissues more rapidly than it is released into the circulation from sites of antibody production.

SUMMARY

The capacity to develop the delayed type of hypersensitivity to diphtheria toxoid and ovalbumin may persist in guinea pigs and rabbits that have received doses of x-ray sufficient to eliminate a detectable antibody response. Larger doses of x-irradiation can prevent development of delayed-type hypersensitivity in rabbits.

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[2] It has been frequently stated that the PCA test detects 0.003 μg. antibody N (7). This figure, however, is derived from studies using only 0.1 ml. of a dilution of a potent antiserum. Undiluted serum, for example, is known to diminish the sensitivity of the test, provided the antibody content of the serum is small (21).

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EXPLANATION OF PLATES

All sections were stained with hematoxylin and eosin. Figs. 1 and 2 show delayed-type skin reactions at 24 hours in x-irradiated guinea pigs. Fig. 3 shows a delayed-type skin reaction at 48 hours in an x-irradiated rabbit.

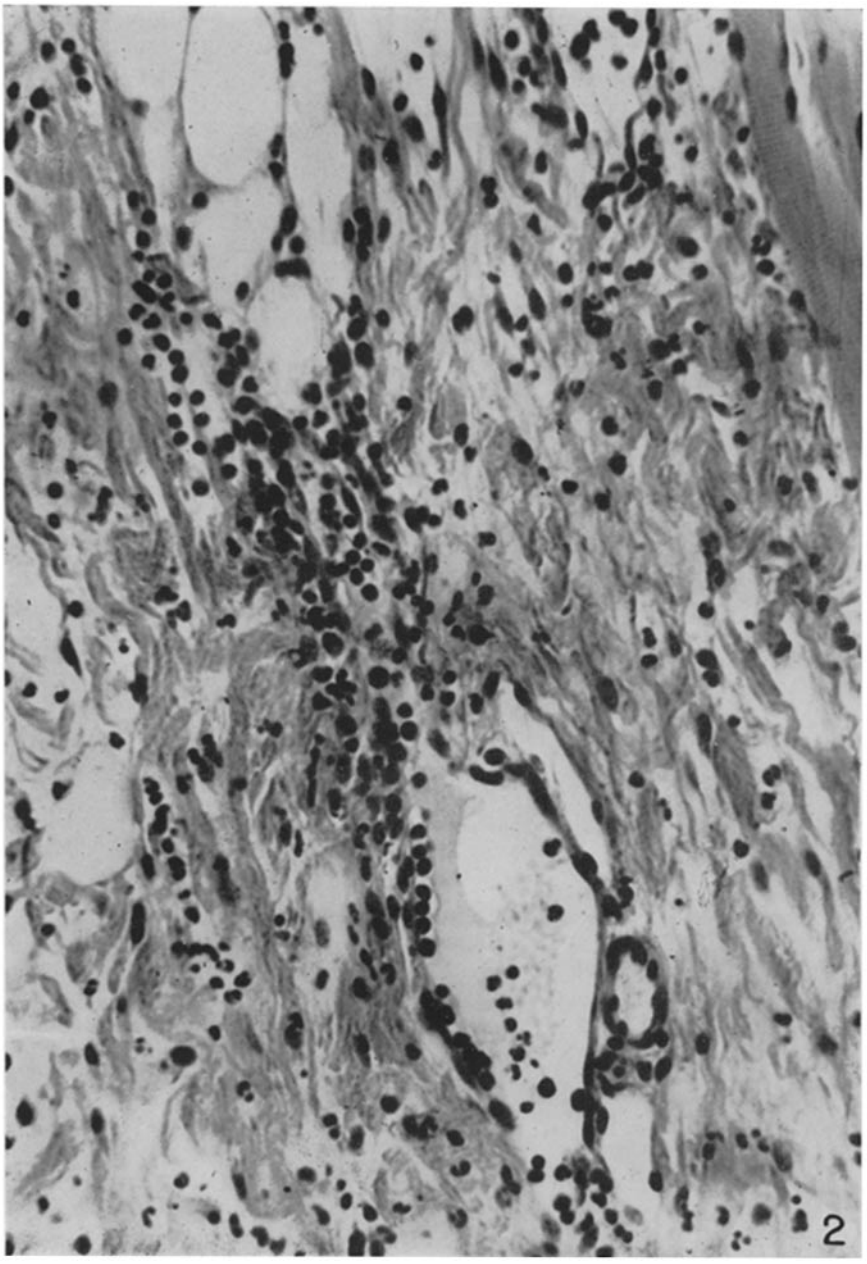
PLATE 3

FIG. 1. There is a cellular infiltrate which is diffuse in the dermis but predominantly perivascular in the subcutaneous tissue. × 70.

(Uhr and Scharff: Delayed hypersensitivity. V)

PLATE 4

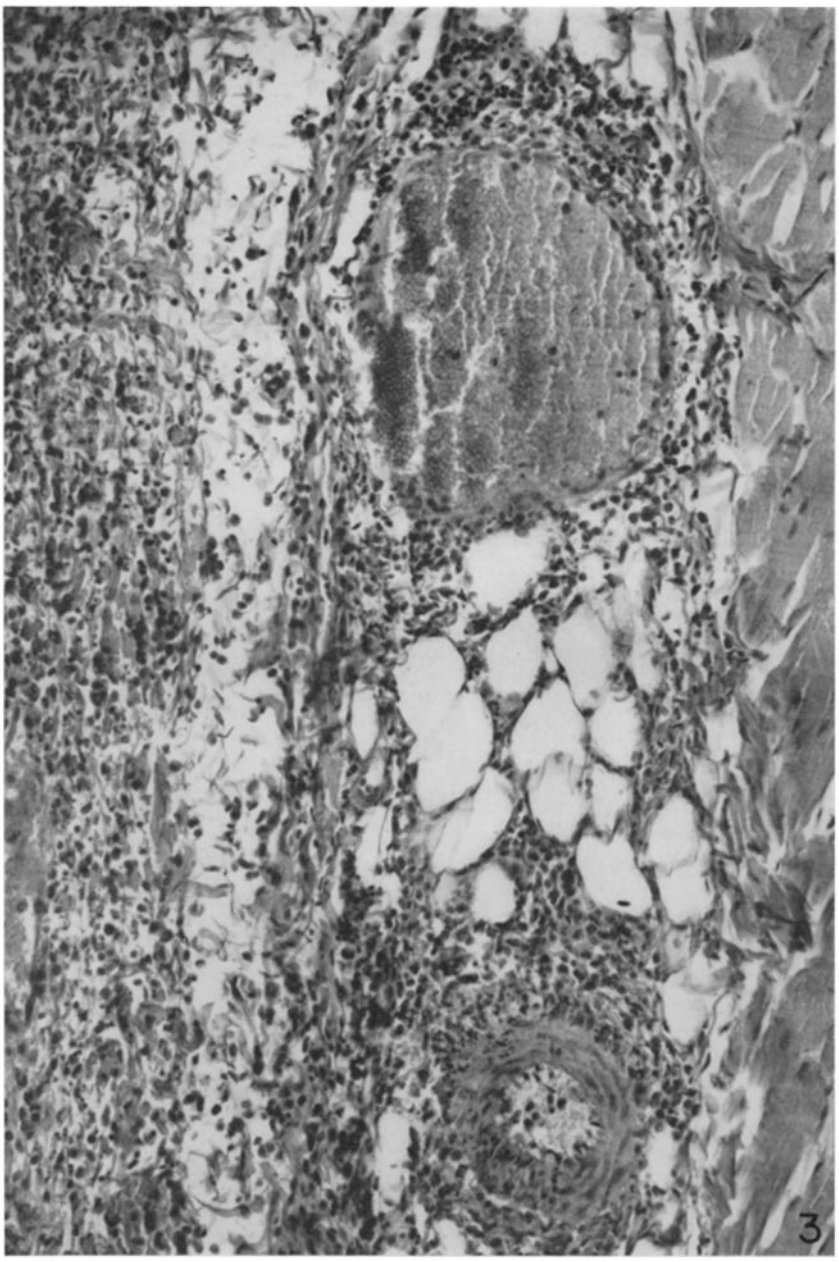
FIG. 2. The inflammatory reaction is more marked in the vicinity of the venule. It consists of histiocytes, lymphocytes, and a small number of neutrophiles. × 500.



(Uhr and Scharff: Delayed hypersensitivity. V)

PLATE 5

FIG. 3. There is a pronounced inflammatory reaction throughout the dermis and subcutaneous tissue. It resembles that seen in Fig. 1 but is more intense. × 200.



(Uhr and Scharff: Delayed hypersensitivity. V)